Towards durable resistance to apple scab using cisgenes

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Towards durable resistance to apple scab using cisgenes

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Educational statement

Dedicated to my beloved parents

Chapter 1

General Introduction

Apple crop

Apple (*Malus* x *domestica*) is one of the important fruit crops of the world. The cultivated apple (*Malus* x *domestica*) belongs to the family Rosaceae, the sub-family Maloideae (Haris et al. 2002). It is the fourth most widely produced fruit crop in the world after bananas, oranges and grapes with a production of 66 million tonnes cultivated on 5 million ha (FAO, 2007). China is the largest producer (43%) with USA being the second largest producer (7%) of apple in the world (FAO, 2007). Apple has many health promoting compounds. It has very strong anti-oxidant activity, inhibits cancer cell proliferation, decreases lipid oxidation, and lowers cholesterol level. Apples contain a variety of phytochemicals, including quercetin, catechin, phloridzin, and chlorogenic acid, all of which are strong anti-oxidants (Boyer and Liu 2004). Apple production has suffered many drawbacks such as apple scab as fungal disease and fire blight as bacterial disease (Gessler and Patocchi 2007) resulting in huge loss in yield.

Apple juvenility is the phase from seed germination untill the first flowering (Jonkers 1971). This period usually lasts four to six years (Webster 2005). This is a serious problem in conventional apple breeding. This can be overcome by physiological or transgenic approaches. The physiological method includes induction of early flowering by putting young seedlings in a climate chamber with high light intensity, high CO_2 concentration and optimal temperature for shoot growth leading to many internodes. This led to flowering of one-year-old trees (Volz et al. 2007). The transgenic approach includes expression of *BpMADS4* gene in apple, which led flowering in invitro shoots thirteen weeks after transformation (Flachowsky et al. 2009).

Self incompatibility is another important problem which restricts fertilization and fruit setting in many tree fruit crops. Self incompatibility is governed by special site S-locus. This S-locus is a gene-complex with highly polymorphic which exists in many alleles. Apple trees display gametophytic self-incompatibility (GSI) (Broothaerts 2003). In GSI, pollen can germinate and penetrate the stigma, but a pollen tube cannot grow completely through the style, if its S-allele is equal to one of the S-alleles present in the style cells (Wertheim and Schmidt 2005). The two S-alleles in the style cells are of equal value (codominant), and thus both involved in affecting pollen-tube growth. They code for the production of enzymes called S-RNases (S for stylar and RNases for ribonucleases) that occur in the intracellular matrix of the transmitting tissue. In apple S-proteins are localized in the intercellular space of the transmitting tissue both in stigma and style (Certal et al. 1999). Self incompatibility can

be over come by developing self fertile apples by silencing S-RNase gene (Broothaerts et al. 2004).

Apple scab

Apple scab is a fungal disease caused by *Venturia inaequalis* (Cke) Wint. In general this fungus takes nutrients through the active and the passive mechanisms. The active mechanism involves movement against concentration gradient which requires the direct input of substantial amounts of metabolic energy, ATP or proton gradients, to drive transportation. The passive mechanism is through diffusion, which does not require carriers. Such nutrients are usually soluble in lipids and can enter hydrophobic membranes, e.g. glycerol and urea (Waites et al. 2001). The pathogen *V. inaequalis* has two distinct phases, a obligate Biotrophic phase from the first ascosporic infection until leaf death or fruit maturity in which it growths subcuticularly on the host and a saphrophytic phase in old leaves and mature fruits where it can invade all the tissue. *V. inaequalis* obtains nutrients through an active mechanism rather than through a passive one (Nicholson and Rahe 2004). The crop loss due to apple scab may be more than 70% of total fruit value (Agrios 2005).

Pathogenesis

V. inaequalis has a hemibiotrophic life cycle characterized by a saprophytic phase in autumn-winter and a parasitic phase in spring-summer. The fungus passes the winter in the old fallen leaves as immature pseudothecia (fruiting body). Each pseudothecium contains 4 genetically different ascospore pairs derived from one meiosis (= 4) followed by one mitosis (4 x 2) resulting in eight ascospores per pseudothecium. Ascospores constitute the primary source of inoculum. Inoculation is brought about through the agency of the wind. For infection to occur, ascospores and conidia have to land on a young leaf or fruits of a compatible cultivar and need a water film to germinate. The ascospores are discharged into the air from the old leaves on the ground. Spore discharge is conditioned by (1) the maturity of the ascospores themselves. (2) The occurrence of rain-periods when the ascospores are mature. (3) Ascospores are also discharged by light. It has been estimated that 45 minutes of wet period at optimum time is enough to discharge eight billion ascospores from the fallen old leaves under a large apple tree. These ascospores require several hours of rainy conditions for germination. Within a few days, the pathogen establishes a food relation with the host (Hesler 1917).

Spore germination: The conidia of *V. inaequalis* germinate readily in water. The conidia have very high specific gravity and on immersion in a drop of water they sink to the bottom of it. At this time conidia are attached to the surface on which it is resting. In a hanging drop, the germ tube continues to grow out as a long thin colorless hypha, but on the leaf desiccation, germ tube comes in contact with the cuticle and proceeds with the formation of "appressorium" (Wiltshire 1915). This penetrates the cuticle. Mycelium grows radially between the cuticle and epidermal cells from the point of invasion, partially dissolving the cuticle. The mycelium of the pathogen on the leaves and fruits, transforms into a stroma, a spore-bearing cushion (Hesler 1917).

Penetration: Penetration of the cuticle and further growth of the fungus must be on young leaves and fruits. When the appressorium is firmly established growth of the hypha into the cuticle takes places, beginning as a small bulge from the centre of the attached disc. The hypha usually grows directly into the cuticle at right angles to the surface of the leaf. It is often enlarged as it enters to attach further. The hypha begins to grow between the epidermal wall and cuticle, forming a plate-like mycelium.

Conidiogenesis: The subcuticular mycelium having been formed in the leaf, the uppermost cells of the stroma starts to enlarge, and push out the cuticle until they burst it open and form the conidiophores from which conidia begin at one end to be cut off (Wiltshire 1915). The conidia mature and will be dispersed to other leaves and fruits, and serve as secondary source of infection. From these secondary infection spots, caused by mycelial growth from the conidia, stromata arise on which a second crop of conidia are produced. This process repeats itself throughout the growing season. As the leaves fall, the mycelium of the organism penetrates all parts of the leaves. Pseudothecia are partially formed during autumn and early winter, and they will remain dormant until spring. At this time growth is resumed and the life-cycle is again started (Hesler 1917). Pathogenesis is visualized in Figure 1. Figure 2 shows infection at the later stage of development of scab on fruits and leaves.

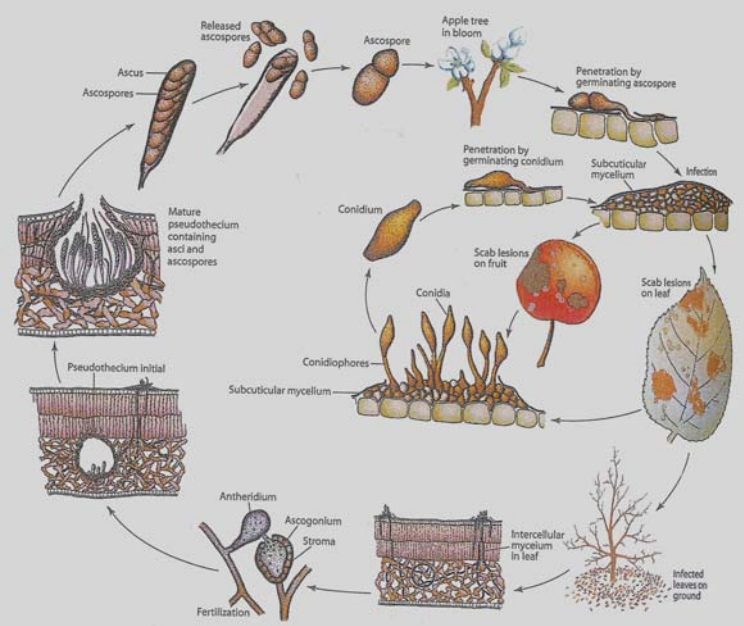

Figure 1. Life cycle of *V. inaequalis* (reprinted from Agrios 1998)

2a 2b

Figure 2. 2a. Scab infected fruits, 2b. Scab infected leaf

Genetic improvement of apple

Most of present day cultivars are susceptible to apple scab disease and fruit growers have to spray 15 times with fungicides in a season. Wild species of *Malus* are resistant to the scab disease (Soriano et al. 2009) and can be good sources of transfer of scab resistance to high quality susceptible cultivars. Thus there is an urgent need for genetic improvement of apples for scab resistance. It can be achieved in three ways, i.e. by making sexual crosses of selected apple genotypes, by mutation breeding, and by addition of genetic material through genetic modification.

Crop improvement through conventional breeding

Conventional breeding of apple aimed at transferring the scab resistance trait is achieved mostly by artificial pollination of selected genotypes with pollen from selected donors. However, there are some bottlenecks concerned with the conventional breeding of apple. Firstly, development of a variety takes a lot of time as the juvenile period is very long i.e. four to ten years (Ibanez and Dandekar 2007). Secondly, during crossing along with the trait(s) of interest other undesirable traits may be introgressed which is referred to as "genetic drag". In order to improve the quality of the genetic material it is necessary to perform sequential crosses with elite material for several generations, because apple is self-incompatible and one cannot perform backcrosses with one high-quality parent (Schouten et al. 2009). In 1914 crosses were made for introduction of resistance to apple scab into commercial apple varieties, using the crab apple *Malus floribunda* 821 as a source of resistance (Crandall 1926). Approximately 85 years after the first cross, *Vf*-cultivars with a good fruit quality were introduced onto the market, e.g. the varieties 'Santana', 'Topaz', 'Florina' (Bakker et al. 1999). So in view of time and genetic drag, improvement of apple through genetic modification is an alternative option.

Crop improvement by mutation breeding

Mutations are changes in the genome that are heritable. Mutations are random. Irradiation with x- and γ rays has been widely used in mutation breeding (Schmidt and Van de Weg 2005). Lapins (1969) was the first to use ionizing radiations to modify the growth habit of McIntosh apple. Mutations are mostly recessive and are important source of variation (Jacobsen and Schouten 2007). Mutations can be seen frequently in varieties like Delicious, Rome Beauty, Cox's Orange Pippin and James Grieve. There are some mutations which are extremely rare or non-existent in varieties like Golden Delicious, Sturmer's Pippin and Granny Smith (Doorenbos 1977). Growth type mutations are also familiar in apple. The wijcik mutant (Starkspur compact Mac) of MacIntosh is very popular for its compact growth, quite normal fruit with good color (Looney and Lane 1984) and it transmits its novel growth habit to a high proportion of its progeny (Lapins 1974) which is very useful in hybridization programs (Lapins 1976). To quote some examples Gala is a variety and its red colored mutants are Galaxy, Mitchgla, Obrogala, etc. Braeburn is a variety and its red colored mutants are Hidala, Joburn, Royal Braeburn etc. Elstar is a another variety and its mutants are Daliter, Elshof, Elstar Armhold etc and red colored mutants are Bel-E1, Red Elstar, Valstar etc. Since mutations are random, targeted mutations could be useful in plant breeding.

Crop improvement through genetic modification (GM)

The second bottleneck of conventional breeding, genetic drag, can be overcome by genetic modification because in that case only wanted gene(s) or alleles are used for introduction (Jacobsen and Schouten 2007). It also obviated the necessity for further sexual crossing. The gene(s) are transferred to the plant through *Agrobacterium tumefaciens* mediated transformation. The selection of transformed cells and regeneration are performed in the presence of an antibiotic like kanamycin. James et al. (1989) developed first transgenic apple to study the rooting of in vitro apple shoots by introducing *nos* and *npt*II genes.

Apple scab resistance genes

Several apple scab resistance genes such as *Vf* (Patocchi et al. 1999), Vr_2 (Patocchi et al. 2004), *Vd3* (Soriano et al. 2009), *Vb* (Erdin et al. 2006), *Va* (Hemmat et al. 2003) and *Vbj* (Gygax et al. 2004) have been identified and mapped on different linkage groups of the apple genome using several independent crosses and progenies by the different research groups. However, only *Vf* has been positionally cloned (Vinatzer et al. 2001). *Vf* proved to be a gene cluster with four paralogs; *HcrVf1* (**H**omologues of ***C****ladosporium fulvum* **r**esistance genes of ***Vf*** region)*, HcrVf2*, *HcrVf3* and *HcrVf4* (Xu and Korban, 2002). At the amino acid level, high degree of homology was observed between *HcrVf1*, *HcrVf2*, and *HcrVf4*. *HcrVf3* was predicted to be a truncated gene. Sequence identity was 84% for the pair *HcrVf1/HcrVf2*, 78% for the pair *HcrVf1/HcrVf4* and 82% for the pair *HcrVf2/HcrVf3* (Xu and Korban 2002). The first transgenic apple plants resistant to apple scab were developed using the *HcrVf2* gene, constitutively expressed by the 35S promoter and *nos* terminator (Belfanti et al. 2004).

Marker free system and Plant transformation

Selection markers are very essential for selection of transgenic tissue. However, the use of selection marker genes has given rise to consumer and environmental concerns over safety (Ramessar et al. 2007). Developing marker-free transgenic plants can address the consumer and environmental concerns to some extent. Krens et al. (2004) discussed different methods to develop marker-free transformed plants. These methods include avoiding the use of any selectable marker (Malnoy et al. 2007; de Vetten et al. 2003) and active marker-removal by site specific recombination using cre-lox system (Dale and Ow 1991), FLP/FRT system (Lyznik et al. 1993), and R/Rs system (Schaart et al. 2004).

In our experiments we used the binary vector pMF1 which is based on the R/Rs system. There are two main advantages of using marker free system. Firstly, removal of marker genes and retaining only cisgenes (own genes), called cisgenesis (Schouten et al. 2006a, 2006b; Schouten et al. 2009), will most likely improve consumer acceptance (Lusk and Rozan 2006). Cisgenesis is defined as the genetic modification of a recipient plant with natural gene(s) from a sexually compatible plant. The gene includes its native promoter and terminator in the normal sense orientation as in the donor plant (Schouten et al. 2006a, 2006b). In cisgenesis, no foreign genes are allowed in the final product. Secondly, the marker-free system allows subsequent stacking of genes using the same selectable marker gene, which was earlier removed by site specific recombination. In this way, multiple genes can be used in gene pyramiding, to obtain broad spectrum and durable resistance to apple scab.

Since GM has many advantages like minimal genetic drag, reduction in juvenile period and stacking of multiple genes (pyramiding), it can be regarded as an attractive option for apple improvement.

Scope of the thesis

In this thesis, many aspects of the development of genetically engineered apples for scab resistance will be discussed. Apple varieties resistant to scab will help growers by reducing production costs and they will help the environment by reducing the number of applications and herewith, the hazardous effects of fungicides.

Different steps are involved in the development of genetically engineered apples for scab resistance using *HcrVf1* and *HcrVf2* cisgenes and intragenes. Different aspects of the development of cisgenic apples are being reviewed in the **Chapter 2**. Isolation of these cisgenes and intragenes, developing transgenic apple shoots, molecular analysis and analysis of transgene copy number of these transformants have been studied and an attempt is made to study the correlation between copy number and gene expression in **Chapter 3**. The transformants were multiplied to get sufficient numbers of each transformation event and they were subsequently micrografted in order to be able to perform resistance tests in the greenhouse. These micrografted transformants were inoculated with monoconidial isolate EU-B05 of *V. inaequalis*. Inserted gene expression was analysed through quantitative RT-PCR and an attempt is made to correlate the resistance and expression levels as is explained in **Chapter 4**. Based on the results of the resistance test and according to some earlier reports on race-specific scab resistance, another experiment was set up using different *V. inaequalis* isolates to check the spectrum of resistance provided individually by *HcrVf1* and *HcrVf2*

(**Chapter 5**). There were reports that new *V. inaequalis* isolates have been evolved that can overcome the resistance of *HcrVf***.** So it is essential to identify new scab resistance genes and combine multiple genes to get durable resistance against scab. The mapping of a newly discovered scab resistance gene, *Vd3*, which was identified while performing a scab resistance test in the greenhouse to map another scab resistance gene has been described in **Chapter 6**.

Finally, all the above described parts will be integrated in the General Discussion in **Chapter 7.** The technique of making plants marker free to make them cisgenic or intragenic, and preliminary results obtained have been described elaborately in this general discussion. Suggestions will be made to select promoters and genes to obtain optimal expression and durable scab resistance.

Chapter 2

Approaches for Development of Cisgenic Apples

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Abstract

Introgression of genetic traits from wild apple germplasm (*Malus* spp.) into commercial apple cultivars is a painstakingly slow process. For e.g. introgression of the *Vf* gene from *Malus floribunda* 821 for resistance to apple scab, caused by the fungus *Venturia inaequalis*, took more than 80 years due to genetic drag and the long juvenile period of apple. In order to speedup the classical breeding, molecular techniques can be applied to enrich existing commercial apple varieties with functional alleles from sexually compatible plants, preventing genetic drag and keeping the genetic makeup of the commercial cultivar. This concept is named "cisgenesis". This paper describes several approaches and considerations for development of cisgenic apples and stacking of genes. Also we provide an overview of isolated alleles from apple available for cisgenesis at the moment and in the near future.

Keywords: *Agrobacterium tumefaciens*, genetic modification *Malus x floribunda*, marker-free, *Venturia inaequalis*

Abbreviations: ***ALS***, *Acetolactate synthase;* **CaMV**, cauliflower mosaic virus; ***GBSSI***, *granule bound starch synthase*; **GFP**, green fluorescent protein; ***GUS***, *β-glucuronidase*; ***hpt***, *hygromycin phospho transferase*; ***HcrVf***, Homologues of *Cladosporium fulvum* resistance genes of the *Vf* region; ***nos***, *nopaline synthase gene*; ***npt*II**, *neomycin phospho transferase* II; **PGIP**, *polygalacturonase inhibiting protein*; ***PPO***, *polyphenol oxidase*; ***R***, *Recombinase*; **Rs**, Recombination site; **RNAi**, RNA interference; **RT-qPCR**, real time quantitative polymerase chain reaction; **T-DNA**, transfer DNA; **ZFN**, zinc finger nuclease; **GMO**, genetically modified organism

Classical breeding

Genetic improvement of apples can be achieved in three ways, i.e. by making sexual crosses of selected apple genotypes, by mutation breeding, and by addition of genetic material through genetic modification.

Classical breeding of apple is achieved mostly by artificial pollination of selected genotypes with pollen from selected donors. The two gametes are then fused into a new genotype without involvement of other genetic sources. Many of the crosses are performed between genotypes producing high quality fruits with genotypes producing lower quality fruits but having interesting traits as disease resistance. However, there are some problems concerned with the classical breeding of apple. Firstly, development of a variety takes a lot of time as the juvenile period is very long, i.e. four to ten years (Ibanez and Dandekar 2007). Secondly, during crossing along with the trait(s) of interest other undesirable traits may be introgressed. In order to improve the quality of the genetic material it is necessary to perform sequential crosses with elite material for several generations. In 1914 crosses were made for introduction of resistance to apple scab into commercial apple varieties, using the crab apple *Malus floribunda* 821 as a source of resistance (Crandall 1926). The progeny of the cross between *M. floribunda* 821 and susceptible cultivars segregated for resistance in a Mendelian 1:1 ratio. The gene putatively underlying this resistance was named *Vf*-gene (*Venturia inaequalis*: *Malus floribunda*). However, the fruits of the resistant parent *M. floribunda* 821 were very small, approximately 1 cm. The apples of the progeny were also small, and did not have the fruit quality that was required for commercial cultivars (Crosby et al. 1990). This was caused by genetic drag: not only the wanted resistance gene was inherited by part of the progeny, but also many unwanted alleles leading to poor fruit quality and other undesirable traits. In order to get rid of the unwanted alleles, subsequent crosses had to be carried out between resistant progeny and susceptible high quality cultivars. About five generations were required to remove most unwanted alleles from *M. floribunda*, yet keeping the desired *Vf*-gene for scab resistance. Approximately 85 years after the first cross, *Vf*-cultivars with a good fruit quality were introduced onto the market, e.g. the varieties ‘Santana’, ‘Topaz’, ‘Florina’ (Bakker et al. 1999).

The first bottleneck, i.e. the long juvenile period, can be solved to some extent by the induction of early flowering. Volz et al. (2009) put young seedlings in a climate chamber with high light intensity, high CO_2 concentration and optimal temperature for shoot growth, leading to many internodes. This led to flowering of one-year-old trees. Grasmanis and Edwards (1974) reported flower initiation in apple trees on MM104 rootstock in the first year

of growth by short exposure to ammonium ions. After selection of the desired genotype, the trees can be vegetatively propagated under normal conditions in the orchard.

Crop improvement using genetic modification

The second mentioned bottleneck for fast breeding is genetic drag. This bottleneck can be circumvented by introduction of the wanted genes or alleles only, by genetic modification, without co-insertion of unwanted genes (Jacobsen and Schouten 2007). An additional advantage is that the genetic makeup of proven cultivars is maintained, as apple is self-incompatible and heterozygous, the genetic makeup of proven cultivars can never be fully restored in the progeny by traditional breeding. However, in case of cisgenesis only one or a few wanted genes are added to an existing cultivar.

Three types of genetic modification can be distinguished depending on origin and organization of the genetic material used to amend the target apple variety, e.g. transgenesis, intragenics and cisgenesis.

Transgenesis

Transgenesis involves transfer of gene(s) or part of the gene(s) from a heterologous organism, e.g. from micro-organisms such as bacteria or viruses or from non-related plant species. James et al. (1989) were pioneers in genetic modification of apple. They developed transgenic apple using a disarmed Ti binary vector and studied the rooting of in vitro apple shoots by introducing *nos* and *nptII* genes. A few examples of transgenesis in apple are the introduction of a *puroindoline* gene from wheat endosperm under the regulation of the cauliflower mosaic virus (*CaMV*) 35S promoter (Faize et al. 2004), the introduction of a *chitinase* gene from *Trichoderma harzianum* under *CaMV35S* promoter (Bolar et al. 2000; Faize et al. 2003), both aimed at achieving apple scab resistance. Szankowski et al. (2003) introduced the *stilbene synthase* gene from grape vine under control of its own promoter or the *polygalacturonase inhibiting protein* (*PGIP*) gene from pear under the *CaMV35S* promoter into commercial apple varieties in order to obtain resistance to fungal pathogens. Norelli et al. (1994) has focused on resistance to fire blight. They introduced the *attacin E* gene from *Hyalophora cecropia* pupae into ‘Malling 26’ apple. This induced a significantly higher level of resistance than non-transformed ‘Malling 26’ in the greenhouse. Markwick et al. (2003) aimed at resistance to the light brown apple moth (*Epiphyas postvittana*), and

introduced genes encoding the biotin binding proteins avidin and streptavidin. They observed a mortality of 80-90% in larvae in the transformants as compared to 14% in control plants.

Several publications report on the use of apple genes in sense or antisense orientation for improvement of apple. This type of genetic modification is often also transgenesis in case for the regulation of transcription heterologous promoter and terminator sequences are used. For example an antisense sequence of the resident apple *polyphenol oxidase* (PPO) gene which is responsible for enzymatic browning of apples, was used successfully to transform apple to reduce the enzymatic browning apples (Murata et al. 2001). Self fertile apples were developed by silencing the *S-RNase* gene which led to the inhibition of expression of the *S-RNase* gene in the pistil resulting in un-arrested self-pollen tube growth, and fertilization (Broothaerts et al. 2004). Flachowsky et al. (2009) described induction of early flowering in apple through transgenesis by inserting the *BpMADS4* gene from silver birch (*Betula pendula* Roth.).

The promoter of the *CaMV35S* gene, which drives transgene expression in plants to a high level and in a constitutive way, is very widely used in transgenesis. Also terminators of the *CaMV35S* gene and of *Agrobacterium tumefaciens* T-DNA genes, such as the *nopaline synthase* gene (*nos*) are being widely applied in transgenesis (Belfanti et al. 2004). First apple transformants using the apple gene *HcrVf2* with *CaMV35S* promoter for resistance against apple scab (*Venturia inaequalis*) were developed by Belfanti et al. (2004). Malnoy et al. (2008) developed apple transformants with *HcrVf1*, *HcrVf2*, and *HcrVf4* separately under control of their native promoters of at least 2 kb lengths, and used the *nptII gene* for selection. Because of this foreign selection gene, the plants were transgenic. Malnoy et al. (2008) described that the *HcrVf1* and *HcrVf2* transformants exhibited partial resistance to apple scab while *HcrVf4* transformants were susceptible. Silfverberg-Dilworth et al. (2005) were the first who studied different lengths of promoters of these *HcrVf* genes through *gus* activity. They reported that 312 or 632 bp for the *HcrVf1* promoter, 288 bp for the *HcrVf2* promoter and 332 bp for *HcrVf4* promoter provide good expression. Apple transformants with different promoter lengths of *HcrVf2* have been developed and studied by Szankowski et al. (2009). They reported that a promoter fragment of 288 bp for the *HcrVf2* gene is sufficient to confer a high level of scab resistance. Even in case the gene to be introduced is originating from the species itself, this may still be transgenesis if selectable marker genes have been introduced together with the gene of interest. Most often the selectable marker genes are of bacterial origin and may code for antibiotic resistances, herbicide resistances, or are giving nutritional advantage to the transformed cells. Gessler and Patocchi (2007) have reviewed transgenesis in apple crop for insect resistance, disease resistance, herbicide resistance and quality aspects like fruit ripening and self incompatibility.

Although transgenesis may speed up the breeding process considerably, it has given rise to three other obstacles: The first obstacle is Intellectual Property (IP). Genes and methods can be patented, and applying genes and methods owned by others can lead to serious financial dependence on the owners of the IP (Graff and Zilberman 2001). The second obstacle is the biosafety regulation. Genetically modified organisms (GMOs) fall under biosafety regulations. This requires extensive studies for safety of the environment, and depending on its usage, also for food and feed. According to a study of Schenkelaars (2008), the costs for market approval of a genetically modified crop have been on average 5.5 M€ in the USA and 6.8 M€ in the EU. Kalaitzandonakes et al. (2007) estimated similar costs. The third obstacle is the acceptance of GM food by consumers. This is especially important in case of consumption of fresh products that are eaten directly without cooking or processing, such as apples. According to inquiries in The Netherlands, only 4% of the consumers were willing to buy and eat transgenic apples, containing genes from micro-organisms or animals (Jan Gutteling, pers. comm.). In case genes were introduced from a plant not belonging to the species of apple (*Malus* x *domestica*), the willingness to buy and eat increased to 20% while 37% was not willing to buy apples with genes from other plant genera. The other consumers were neutral. The willingness to buy and eat increased further to 35% when only apple genes were used for the genetic modification of apples. When the consumers received information from an independent organization about the genetic modifications, then the willingness to buy apples with own genes only, increased to 47%. 17% were still not willing to buy these apples, and the remaining 36% was neutral (Jan Gutteling, pers. comm.). This shows that the source of the genes has a strong impact on willingness to buy. Lusk and Rozan (2006) showed this also in an inquiry in the USA and France. Therefore the attention for genetic modification using ‘own genes only’ has increased, as is elaborated in the following paragraphs.

Intragenics / Intragenesis

The term intragenic was first used by Nielsen (2003). As the name suggests, intragenics means transfer of functional sequences “within the genera” by genetic transformation. Rommens et al. (2007) defined an intragenic plant as a genetically modified plant that only contains genetic elements derived from within the sexual compatibility group of that plant. Intragenics allows the generation of new combinations of donor DNA sequences. When e.g. the coding sequence of a certain gene from *Malus* is combined with a promoter of a different apple gene and subsequently used in genetic modification of apple, this is termed intragenics. By making new combinations of regulatory sequences and coding sequences one can alter

the expression of the gene of interest to a desired level and pattern. The techniques of gene silencing involving the use of RNA interference (RNAi) and the use of anti-sense strands of resident gene sequences can be considered as examples of intragenics where the orientation of the sequences is not a critical factor (Russel and Sparrow 2008).

As new combinations of regulatory sequences and coding sequences can be made in intragenic plants, this may lead to gene expression levels and expression patterns that are not present in nature or non-GM breeding germplasm of *Malus.* From this point of view, intragenic plants may show phenotypic traits that are not feasible through classical plant breeding methods. Generally, plants from classical breeding are regarded as the baseline for GMO Regulations. As intragenic plants may deviate from this baseline, it could be argued that novel risks may emerge compared to the baseline, requiring additional biosafety tests for market approval (Schouten and Jacobsen 2008).

Cisgenesis

In order to take into account both consumers' attitudes and staying as close as possible to classical breeding, possibly leading to exemption from the GMO regulation, a strict case of intragenics was developed, i.e. cisgenesis. Schouten started to develop the cisgenic approach in 1999 as a result of discussions between ethicists, social scientists and plant scientists (Iversen 2000; Jochemsen 2000). The term 'cisgenesis' was invented by Jochemsen and Schouten in 2000 (Jochemsen 2000), when Schouten and Schaart developed strawberry plants with resistance to greymould, caused by *Botrytis cinerea,* using a native *PGIP* gene from strawberry (Schaart 2004) but combined with a promoter of strawberry *Expansin* gene. The onset of cisgenesis was several years before the development of intragenics by Nielsen (2003) and Rommens (2004).

After the development of strawberries with its native *PGIP* gene, Schouten and Jacobsen further developed the concept of cisgenesis and made the definition stricter compared to intragenics. Cisgenesis is defined as the genetic modification of a recipient plant with natural gene(s) from a sexually compatible plant. The gene includes its native promoter and terminator in the normal sense orientation as in the donor plant (Schouten et al. 2006a, 2006b). In cisgenesis, no foreign genes are allowed in the final product.

There are two main differences between cisgenesis and intragenics. Firstly regarding the regulatory elements: Cisgenesis uses the native regulatory elements belonging to the target gene like promoters, introns and terminators. The inserted gene is an exact DNA copy of the complete natural gene. However, in case of intragenics new combinations of coding sequences and promoters are used. Intragenics has no requirements regarding introns or terminators, the only requirement is that the genetic elements are taken from within the

sexual compatibility group. Secondly, unlike intragenics, cisgenesis does not apply the RNAi or antisense approach based on novel combinations of genetic elements (Schouten and Jacobsen 2008).

In the case of cisgenesis, where no novel combinations between coding sequences and regulatory elements such as promoters are made, but only complete genes are used from sexually compatible plants, the gene expression levels and patterns should be similar to expression of plants from classical breeding. If that is the case, it can be argued that the phenotypic traits of such plants can also be obtained through classical breeding. If no novel traits are introduced compared to classical breeding, it can be argued that such plants are as safe as plants from classical breeding, and should be exempted from the GMO regulation (Schouten et al. 2006a, 2006b; Jacobsen and Schouten 2007; Schouten et al. 2009). A cartoon for the different breeding strategies is given in Figure 1.

Methods for the development of cisgenic apples

Transformation with *Agrobacterium*

Cisgenic apples are developed through the process of genetic modification. In apple, like in many other crops, *Agrobacterium*-mediated transformation is the preferred method for gene transfer. After the introduction of genes into the plant cell genome, these particular cells with their newly acquired traits should be preferably multiplied and regenerated into entire plants. In order to ensure that all the regenerants contain the desired genes-of-interest, selectable marker genes are usually added as well. Within the concept of cisgenesis it is imperative that such genes, when derived from other, sexually non-compatible organisms, are not present in the final product.

Usage of marker genes

Use of selectable marker genes is a very important element in plant transformation (Komari et al. 2007). Some of the most common markers are genes conferring resistance to antibiotics such as kanamycin or hygromycin, or tolerance to herbicides, e.g. phosphinothricin or glyphosate. Kanamycin resistance has been most frequently used in transformation of many dicotyledonous plants, including apple. This selection gene has a bacterial origin and cannot be left behind in cisgenic apple plants.

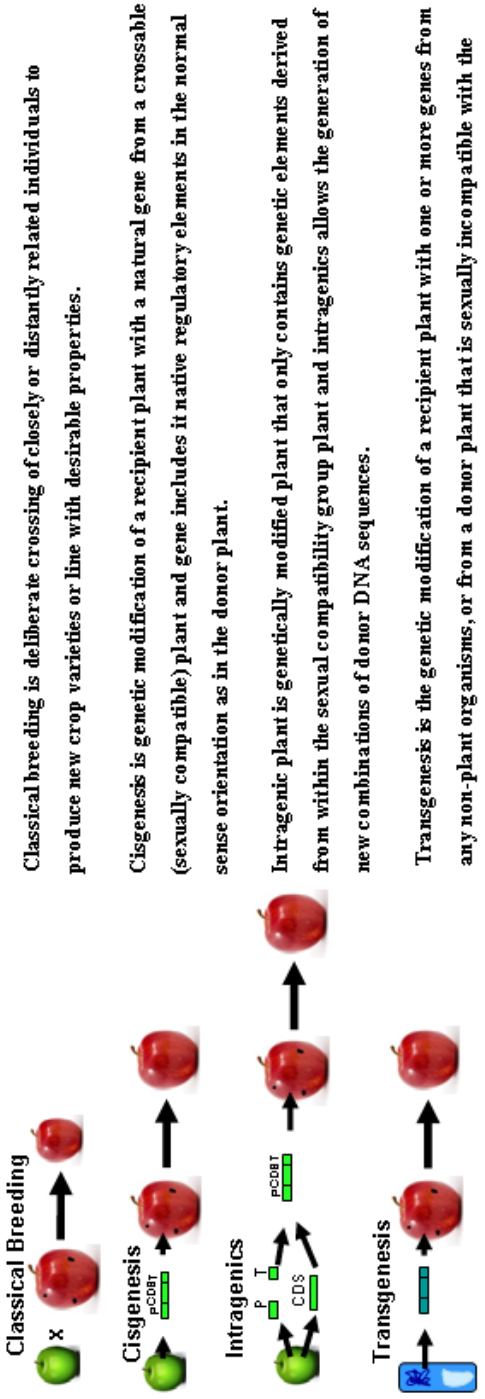

Figure 1. Different breeding strategies for apple. P-Promoter, CDS-Coding sequence, T- Terminator.

Methods to generate marker-free transformed plants

Krens et al. (2004) discussed different methods to develop marker-free transformed plants. These methods include a) avoiding the use of any selectable marker; b) co-transformation; c) active marker-removal by recombination. Approaches a) and c) have been used in apple and will be dealt with in the following paragraphs. Co-transformation of plants with both a T-DNA vector carrying the selectable marker gene and a T-DNA vector containing the gene of interest might result in unlinked integration events, which can be segregated in the progeny. However, this requires sexual crossing which is not preferable in apple as it will destroy the genetic make-up of the cultivar. Avoiding the segregation step could be tried by co-transferring two T-DNAs, one carrying a marker gene and the other containing the gene-of-interest, followed by a selection step. The aim is that only the T-DNA with the gene-of-interest will integrate, while the other T-DNA with the selection gene will only be transiently expressed and active without integration into nuclear or plastid DNA (Rommens et al. 2004). This method appeared to be unsuccessful in apple (Giovanni Broggini, pers. comm.).

Transformation without a selection gene

Transformation without a selection gene will lead to the regeneration of many shoots originating from both untransformed and transformed cells. de Vetten et al. (2003) demonstrated that marker free plant transformation is possible in the vegetatively propagated species potato, using PCR afterwards for selecion of the transformed plantlets. They transformed with an antisense construct for silencing the granule bound starch synthase (GBSSI) gene, using an additional upstream inverted copy of its 5'region to obtain amylose free potatoes. Malnoy et al. (2007) employed this technique of transformation without selection in apple. Due to the fact that usually only a few cells within a tissue are successfully transformed most of the regenerated shoots will be non-transformed plantlets. In order to identify the genetically modified clones, all plantlets must be checked for the presence of the gene(s) of interest by PCR.

Because of the lack of selection pressure, there is a considerable risk of obtaining chimeric plants that only partly consist of genetically modified tissue.

Transformation with a foreign selection gene and elimination of the selection gene

This method makes use of site-specific recombination systems for removal of the selection gene after the transformation step (Dale and Ow 1991). The method comprises integration of T-DNA which carries a selectable marker gene. This marker gene is flanked by two recognition sequences specific for a recombinase whose activity can be controlled. Following successful T-DNA integration and selection of regenerants, the recombinase is activated and it excises the selectable marker gene.

There are several systems available consisting of a gene coding for a site-specific recombinase enzyme and specific recombination sites flanking the undesired sequences *viz*. the *cre-lox* system (Dale and Ow 1991), the R/Rs system (Schaart et al. 2004) and the FLP/FRT system (Lyznik et al. 1993). In the R/Rs system, the site-specific *Recombinase* (*R*) and the recombination sites (Rs) originate from the yeast *Zygosaccharomyces rouxii.* These Rs are 59-82 bp in length and consist each of three direct imperfect 12 bp repeats and one inverted repeat. The two Rs should be in the same orientation. After recombination a single Rs is left. The activity of *R* is inhibited at first by the combination with a ligand-binding domain. The Recombinase activity can be induced by the addition of dexamethasone (Schaart et al. 2004). The first report on active removal of a selectable marker gene was published in 1991 by Dale and Ow. They used the *cre*/lox site-specific recombination system and demonstrated in tobacco that the *hpt* selection gene flanked by two lox sites introduced in a primary transformation could be removed by a second transformation step with a binary vector carrying the *npt*II gene together with the recombinase *cre* gene. More details on the different systems including induction steps are given by Schaart et al. (2009). We use this technique for development of cisgenic apples.

A general consequence of the application of the mentioned recombination systems is that always a single recombination site, which is of foreign origin, is left behind in the recipient genome. Such recombination sites are usually 34 and 82 bps long in lox and Rs respectively and are expected not to be functional without a second recombination site and its corresponding specific recombinase.

Chimeras

Occurrence of chimeras

A chimera consists of sectors or tissues that differ in genetic constitution. Chimeras can arise from regeneration from multiple cells (transformed and untransformed ones) or after transformation of one cell in an already existing multicellular shoot meristem or

embryogenic region. Gahan and George (2008) reported that in *P. sativum* transformed with the *gus* gene, the chimeric shoots are likely to be formed from at least one transformed and one untransformed cell. This will result in a plant that contains layers or sectors of transformed tissue. Chimerism can be a problem especially in vegetatively propagated plants, where no fixation through a sexual phase occurs (Ahuja 1992). Identification of chimeras after genetic modification and subsequent regeneration is normally very difficult and generally not done. Chimerism in the protocol for the development of cisgenic apples can occur in a form where a plant is built up of non-transformed and transformed cells. Chimerism may also occur after the recombination, the chimeras will consist of both marker-free cells as well as of marker-containing, non-recombined cells and will be identified as non-recombined upon molecular characterization. Those individuals will not be used further and be discarded. Still, careful screening of putative marker-free plants is required.

A final possibility of chimerism is loss of function mutation of the transgene, intragene or cisgene itself. This type of chimerism will also be found in marker aided selection of GM plants. Russel et al. (1992) transformed *Arabidopsis* with the gene *acetolactate synthase* (*ALS*) encoding sulfonylurea (SU) herbicide-resistance. They placed the gene between loxp sites. After the recombination event they observed chimerism in the F_1 progeny of lox/ALS/lox/gus and cre/hpt plants. Some plants were completely sensitive to sulfonylurea in the stem callus assay, as the *ALS* gene was excised, but showed varying percentages of resistant or chimeric (intermediate resistant) progeny, indicating chimerism. In the cisgenic approach, all cells of a cisgenic plant need to be without the marker genes.

Chimerism can be minimized by optimization of the primary regeneration process or by subjecting the confirmed transformant to re-regeneration, grafting or budding and again testing for respectively the presence or absence of the gene-of-interest and the selectable marker gene. This can be a good method to identify chimeras in perennial crops like apple which in natural situation takes years together. In our experiments we have used the marker free system pMF1 (Schaart et al. 2004) and we have not experienced any kind of chimerism or somaclonal variation. If the binary vector system is not efficient, it may give rise to chimerism.

Identification of chimeras

Chimeras can be identified by the use of reporter genes, e.g. encoding *β-glucuronidase* (*gus*) (Jefferson et al. 1987), green fluorescent protein (GFP) (Chalfie et al. 1994), *luciferase* (Koncz et al. 2005), red fluorescent protein DS-RED E-5 (Mirabella et al. 2004) or *MdMYB10* (Espley et al. 2007). These reporter genes can then be placed between

recombination sites. Transformants can be evaluated for chimerism by positive selection for uniform production of blue color (*gus*), green or red light emission (GFP), and red color (*MdMYB10*) respectively after transformation and uniform absence of these colors after recombination in cisgenic plants (Schaart et al. 2009). Faize et al. (2009) demonstrated that quantitative real time PCR (RT-qPCR) can also be a reliable molecular method to identify and quantify the chimeras in transformed apricots, using genomic DNA. This was based on quantitative real time PCR amplification of *npt*II as well as internal control (*actin*), used to normalize the quantity of *npt*II.

Stacking genes/alleles

One of the advantages of marker-free and therefore also of cisgenic apple plants is the possibility to add more cisgenes to plants that already have obtained cisgenes earlier. In these secondary genetic modifications the absence of selectable marker genes allows the repeated use of the selection system that was optimized for apple.

Gene stacking can be done in three ways, 1) inserting all the genes of interest at once, so stacking the genes in a binary vector prior to the transformation event, 2) inserting the genes of interest consecutively, one after the other by retransformation or 3) by crossing two different transgenic lines and checking the progeny for the desired combination of genes. The latter option using sexual crossing is not well-suited for apple as it destroys the genetic make-up of elite genotype.

Retransformation was first described by Dale and Ow (1991). They transformed tobacco introducing the *luciferase* reporter gene and the selectable marker gene *hpt* for hygromycin resistance placed between two lox sites. Selection was on hygromycin. Established primary transgenic tobacco was retransformed introducing the *cre* gene together with the *npt*II gene for subsequent kanamycin selection. In this way they showed that selectable marker genes can be effectively eliminated by a system based on recombination and stacking of genes, i.e. *luc* and *cre* and *npt*II, is possible. However they removed the *cre-npt*II locus through self pollination and segregation and retained only *luc* gene. Stacking of the genes in plants without sexual crossing was also reported by Sugita et al. (2000) when they transformed *uidA* and *nptII* genes in primary transformation with *ipt* as selectable marker and then they excised the *ipt* gene which was between two recombination sites. They did secondary transformation of GFP by using same *ipt* gene as selectable marker. After secondary transformation and regeneration they were able to trace back *npt*II, *uidA* genes and GFP in the transformants. Retransformation with the same selection gene i.e. adding new genes to a

transgenic line already equipped with other transgenes after removal of the selection gene, has not been attempted yet. Also transformation with binary vector containing both the old and new genes and transforming a crop with this new stacked construct, has not been done yet. Both require the full process of transformation and marker elimination and will take comparable amounts of time. Still, when introducing multiple genes at once, one will have to look for those cisgenic plants in which all genes will perform as necessary for the desired phenotype.

Available genes for the generation of cisgenic apples

Cisgenesis allows stacking of new desired genes coding for interesting traits, whenever they will become available, in elite cultivars. In this way, multiple traits can be brought together relatively fast by genetic modification. For e.g. at present, for apple scab resistance, the race specific resistance genes *HcrVf1* and *HcrVf2* have been isolated from apple (Vinatzer et al. 2001) and applied for cisgenesis. Once introduced in elite, originally susceptible apple cultivars, stacking can be done later with other apple scab resistance genes, providing resistance to other isolates of the pathogen *Venturia inaequalis*. An example of such a resistance gene is the *V25* gene (Soriano et al. 2009) that provides a broad spectrum for resistance against apple scab disease. By means of map based cloning, one Bacterial Artificial Chromosome (BAC) clone carrying three candidate genes for *V25* has been identified. These candidate genes will be functionally analyzed. Other examples of resistance genes to apple scab are the nearly isolated and functionally analyzed genes *Vr2* (Patocchi et al. 2004) and *Vm* (Patocchi et al. 2005). By stacking these resistance genes, a broad spectrum of resistance to apple scab can be realized leading to an increase in durability of resistance.

Other alleles may be added such as the apple transcription factor *MdMYB10* (Espley et al. 2007). This gene has a repeat in the promoter region onto which the *MdMYB10* protein binds, thus upregulating its own expression (Espley et al. 2009). This transcription factor regulates the anthocyanin biosynthesis pathway leading to production of anthocyanins and red coloration throughout the plant. This red coloration can be seen not only in the skin but also in the flesh of the fruit and even in the young plantlets after transformation and regeneration. This red color can also be helpful for selection of cisgenic plantlets even without antibiotic selection (Figure 2). These red-fleshed apples have enhanced anti-oxidant capacity which may be health beneficial to consumers. The *MdMYB10* gene may be stacked along with scab resistance.

In view of the upcoming whole genome sequence of the apple cultivar 'Golden Delicious' (Ricardo Velasco, pers. comm.), numerous genetic mapping studies (Baldi et al. 2004; Calenge et al. 2005; Gardiner et al. 2009) and the present EST database for apple (Newcomb et al. 2006) the number of isolated alleles that are functionally characterized and readily available for cisgenesis, will increase faster and faster. In the near future, a large number of genes will be isolated and made available for cisgenesis, such as alleles for fire blight resistance, columnar type architecture of the apple tree, and for health beneficial compounds.

Figure 2 Apple transformation with solely the apple *MdMYB10* gene**.** Cisgenic plantlets that are grown in the dark can be selected, based on their red color. For this transformation the *MdMYB10* gene (Espley *et al*. 2007) under control of its native promoter (Espley *et al.* 2009) was inserted into leaf material of 'Gala'. No additional gene was used for e.g. selective advantage. This picture shows regeneration of callus tissue that received the *MdMYB10* gene through *A. tumefaciens* mediated gene transfer. Picture: Aranka van der Burgh (H.J. Schouten et al, in prep.).

Future targets

The draw back of the transformation is the random insertion of the cisgene into the genome. This may lead to change in the expression of the gene compare to native expression. Targeted insertion or allele replacement has been already applied to higher plant with success (Terada et al. 2002). Shaked et al. (2005) showed the homologous recombination-mediated integration of DNA segment into chromosomal target sequence by expressing the yeast *RAD54* gene in *Arabidopsis*. Zinc finger nucleases (ZFNs) proteins as molecular scissors can also be employed in targeted integration or allele replacement by homologous recombination (Durai et al. 2005). Mostly single genes were targeted by homologous recombination. However the applicability of such methodology to apple has not been yet tested.

Concluding remarks

The main driver for genetic modification (GM) in apple is the painstakingly slow breeding process of classical breeding, due to the long juvenile period and genetic drag. GM is much faster, as it reduces genetic drag to a minimum. Moreover the genetic makeup of elite cultivars can be maintained. The second driver is the exponentially increasing number of functionally analyzed genes. The number of well characterized genes of apple will increase soon, especially because of the unraveling of the whole genome sequence of the apple cultivar 'Golden Delicious'.

There are also obstacles for GM: Not all forms of GM are accepted by consumers, especially in case of fresh fruits, such as apple. The willingness to buy apples with foreign genes is low. However, in case only apple genes are used for the genetic modification like in cisgenesis, the willingness to buy and eat increases strongly around the globe. Moreover, GM technologies are present that allow introduction and stacking of own genes without leaving any foreign genes behind.

The second obstacle for GM of apple for commercial purposes is the costly approval procedure of GM trees and apples for proving safety for environment and food. In case of cisgenesis, no novel traits are introduced compared to classical breeding. Solely well-known apple alleles are added to well-known apple cultivars, and consequently cisgenic apples are at least as safe as classically bred apples. Therefore cisgenic apples should be approved in a timely and cost effective manner, preferably by means of exemption from the GMO regulations (Schouten et al. 2006a, 2006b).

Another future challenge is the maintenance or repair of the epigenetic state of alleles, such as methylation pattern. A PCR reaction and cloning step strips of all methylation of the promoter and coding region. Also other non-native epigenetic changes may occur during isolation and insertion. This may have an effect on the expression pattern. In spite of these possible effects, the level of biosafety control of cisgenic apples is higher compared to classically bred apples.

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Chapter 3

Isolation of apple *HcrVf* genes and development of transgenic apple plants

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Abstract

Most of the present day apple cultivars are susceptible to apple scab, a fungal disease which is caused by *Venturia inaequalis*. Fruit growers spray on an average 15 times in one year to control the disease. Apple scab resistance genes, *HcrVf1* (**H**omologues of ***C****ladosporium fulvum* **r**esistance genes of ***Vf*** region) and *HcrVf2* were isolated including their native promoter, coding and terminator sequences. The native gene promoters for the two *HcrVf* genes were tested for expression and phenotype in two lengths (*HcrVf1* short promoter (SP)-312 bp, *HcrVf1* long promoter (LP)-1990 bp; *HcrVf2* short promoter (SP)-288 bp, *HcrVf2* long promoter (LP)-2000 bp). As a non-native, highly active promoter, the apple rubisco promoter (P_{MdRbc}) (1600 bp) was tested for directing expression of the two *HcrVf* genes. Apple transformants were tested for expression by quantitative RT-PCR (qRT-PCR) and were analyzed for transgene copy number through Southern hybridization. The results of qRT-PCR showed that in *HcrVf1*, expression driven by LP was significantly higher than by SP, whereas in *HcrVf2* there was no significant difference between SP and LP with respect to expression levels. Rubisco proved to give the highest expression of both *HcrVf1* and *HcrVf2*. Most of the apple transformants were having a single transgene insert and only few had obtained two transgene copies. We did not observe any clear correlation between transgene copy number and gene expression.

Introduction

Apple (*Malus* x *domestica*) is one of the important fruit crops of the world. Most of the present day apple cultivars are susceptible to apple scab which is caused by the fungus *Venturia inaequalis*. Fruit growers spray on an average 15 times in one year to control the disease (Patocchi et al. 2004). This leads to a substantial increase in production costs, may burden the environment and may pose health concerns for consumers (MacHardy 1996).

Crop improvement through classical breeding was initiated in the early 20th century. In 1914 crosses were made for introduction of resistance to apple scab into commercial apple varieties, using the crab apple *Malus floribunda* 821 as a source of resistance (Crandall 1926). There are three main bottle necks in the classical breeding of apple. Firstly, development of a variety takes a lot of time as the juvenile period is very long, i.e. four to ten years (Ibanez and Dandekar 2007). Secondly, during crossing along with the trait of interest other undesirable traits may also get introgressed which is referred to as genetic drag. To get rid of this genetic drag it is necessary to perform sequential crosses between resistant progeny and other high quality cultivars for several generations (Schouten et al. 2009). Back-crosses with the same elite cultivar are not possible because of self-incompatibility, the third bottle-neck.

Crop improvement through cisgenesis is another option for genetic improvement of apple. Several apple scab resistance genes such as *Vf* (Patocchi et al. 1999), *Vr$_2$* (Patocchi et al. 2004), *Vd3* (Soriano et al. 2009), *Vb* (Erdin et al. 2006), *Va* (Hemmat et al. 2003), *Vbj* (Gygax et al. 2004) have been identified and were mapped on different linkage groups of the apple genome. However, only the *Vf* gene has been positionally cloned and proved to be a gene cluster with four paralogs namely *HcrVf1, HcrVf2, HcrVf3* and *HcrVf4* (Xu and Korban, 2002). First, transgenic apple plants resistant to apple scab were developed using the *HcrVf2* gene which was constitutively expressed by the Cauliflower mosaic virus 35S (*CaMV35S)* promoter and *nos* terminator (Belfanti et al. 2004). Later, apple transformants with *Vfa1* (which is the same as *HcrVf1*) and *Vfa2* (which is the same as *HcrVf2*) using native promoters and terminators for expression were developed by Malnoy et al. (2008). They used a 2 kb promoter for both *Vfa1* and *Vfa2*.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is an abundant protein in plants (Ellis 1979) and rubisco protein contents were correlated with total rubisco small subunit (RbcS) mRNA levels in rice (Suzuki et al. 2009). Using the promoter and terminator of rubisco small subunit (*rbcS1*) of chrysanthemum, expression levels of 7 to 8 fold those provided by the constitutively expressed cauliflower mosaic virus (*CaMV*) 35S promoter

were obtained (Outchkourov et al. 2003). The combination of the apple rubisco promoter (P_{MdRbc}) and terminator (T_{MdRbc}) with the *gus* reporter gene resulted in similar expression levels compared to the *CaMV35S* promoter (Schaart et al. 2010b).

In this study we report on the isolation of the *HcrVf1* and *HcrVf2* genes together with their native promoters in two lengths (short and long) and their native terminator. As a reference, the two *HcrVf* genes were combined with regulatory sequences of the apple rubisco gene. To study the effect of the different promoters on expression, the *HcrVf* constructs were introduced in apple. The correlation between transgene copy number and expression was also investigated.

Materials and Methods

Gene amplification

Preparation of *HcrVf* constructs with native regulatory elements

An apple BAC library was constructed from the genomic DNA of elite breeding line 1980-015-025 which harbors several resistance genes such as *Vf, Vd3* (Soriano et al. 2009) and *V25* which is presently being mapped and cloned in our laboratory. The BAC library construction was carried out as previously described by Rouppe van der Voort et al. (1999). The BAC library consisted of 288 BAC pools with each BAC pool comprising of 384 BAC clones. Primers which were described by Xu and Korban (2002) and were used to identify positive BAC pools for the *HcrVf1* and *HcrVf2* genes. The primers are mentioned in Table 1.

Individual positive BAC clones were identified from positive BAC pools for *HcrVf1* and *HcrVf2*. In our BAC library *HcrVf1* and *HcrVf2* genes were identified on separate BAC clones. Two BAC clones were identified that contained *HcrVf1* and two others containing *HcrVf2* genes. One BAC clone containing *HcrVf1* and one containing *HcrVf2* were sequenced at GATC Biotech AG (Konstanz, Germany). Sequenced contigs were aligned with the published *HcrVf1* (Gene bank accession number AY397723) and *HcrVf2* (Gene bank accession number AJ297740) sequences using the software SeqMan in DNASTAR® version 7.0. On the basis of the recommendations by Silfverberg-Dilworth et al. (2005) sequences upto 312 bp and 288 bp upstream of the transcription start site were used as short promoters for *HcrVf1* and *HcrVf2,* respectively. Similarly, sequences up to 480 bp and 437 bp downstream of the stop codon were used as terminators for *HcrVf1* and *HcrVf2,* respectively. Sequences of 1990 bp and 2000 bp upstream of the transcription start site were used as long promoters for *HcrVf1* and *HcrVf2,* respectively, and terminators were the same

as in case of SP*HcrVf1* and SP*HcrVf2*. Thus, the gene sequences for short promoter *HcrVf1* (312 bp) (SP*HcrVf1*), long promoter *HcrVf1* (1990 bp) (LP*HcrVf1*), short promoter *HcrVf2* (288 bp) (SP*HcrVf2*) and long promoter *HcrVf2* (2000bp) (LP*HcrVf2*) were obtained.

The fragments were amplified using Phusion® DNA polymerase (Finnzymes, Espoo, Finland) and BAC DNA as template. The primers used were extended with *AscI* and *MluI* restriction site sequences for *HcrVf1* at 5' and 3' ends respectively, and *AscI* and *PacI* restriction site sequences for *HcrVf2* at 5' and 3' ends respectively. The primers are listed in Table 1. The PCR reaction includes 5X Phusion® HF buffer, 5mM dNTPs, 10µM forward and reverse primers (each), 0.2 U Phusion® DNA polymerase making a total volume of 20 µl. The PCR conditions were as follows: 98° C for 30 seconds, followed by 35 cycles of 98° C for 10 seconds, 56° C for 30 seconds and 72° C for 4 minutes and final extension at 72° C for 10 minutes to generate whole gene amplification of *HcrVf1* and *HcrVf2*. The full gene amplification of *HcrVf1* and *HcrVf2* with their native promoters and terminators has been depicted in Figure 4. Fragments were cloned in pGEM-T easy vector (Promega, Madison, USA) and their identity was confirmed by sequencing to make sure that the complete gene is free of PCR-errors. Subsequently, these fragments were subcloned into the binary, marker-free vector pMF1 (Schaart et al. 2010a) in the multiple cloning site using *AscI* and *PacI* restriction sites. The vector map of pMF1 is given in Figure 2.

Preparation of *HcrVf* constructs with apple rubisco regulatory elements

P_{MdRbc} and T_{MdRbc} sequences were amplified using sequence specific primers (Schaart et al. 2010b). Sequence amplification was done using primers with restriction site sequences of *PacI* and *XmaI* at their 5' and 3' ends respectively for P_{MdRbc} and *KpnI* and *AscI* at 5' and 3' ends respectively for T_{MdRbc}. P_{MdRbc} and T_{MdRbc} sequences were combined into a vector so that they were separated by *XmaI* and *KpnI* and flanked by *PacI* and *AscI* respectively. *HcrVf1* and *HcrVf2* genes were amplified using gene specific primers (Table 1) giving restriction sites *XmaI* at the 5' end and *KpnI* at 3' end and cloned in pGEM-T easy vector. These sequences were confirmed by sequencing to make sure that the sequences are PCR-error free, they were excised from the pGEM-T easy vector using *XmaI* and *KpnI* and subsequently subcloned into the P_{MdRbc} and T_{MdRbc} construct. Then the P_{MdRbc} – *HcrVf* – T_{MdRbc} fragment was subcloned as *PacI* - *AscI* fragment into the destination vector pMF1 (Schaart et al. 2004).

The resulting pMF1-derivatives (*HcrVf* genes with native and rubisco regulatory elements) were transformed to the supervirulent *Agrobacterium tumefaciens* strain AGL0 (Lazo et al. 1991). The gene constructs used in plant transformation are given in Figure 1.

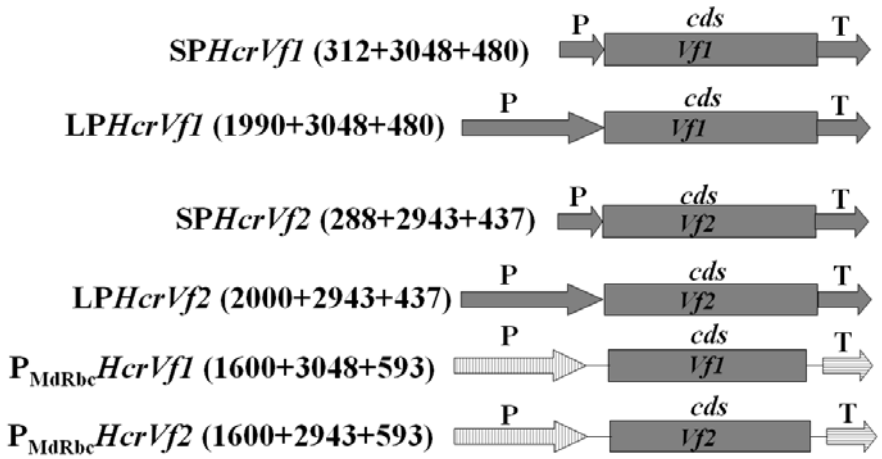

Figure 1. Constructs used for plant transformation. P-Promoter, cds- coding sequence, T- Terminator. The numbers in parentheses indicate the lengths of promoter, coding sequence and terminator in basepairs. Vertical stripes represent apple rubisco promoter and horizontal stripes represent apple rubisco terminator. All LP and SP constructs represent stretches cloned as a whole (cisgenes); the P_{MdRbc} constructs represent new combinations (intragenes).

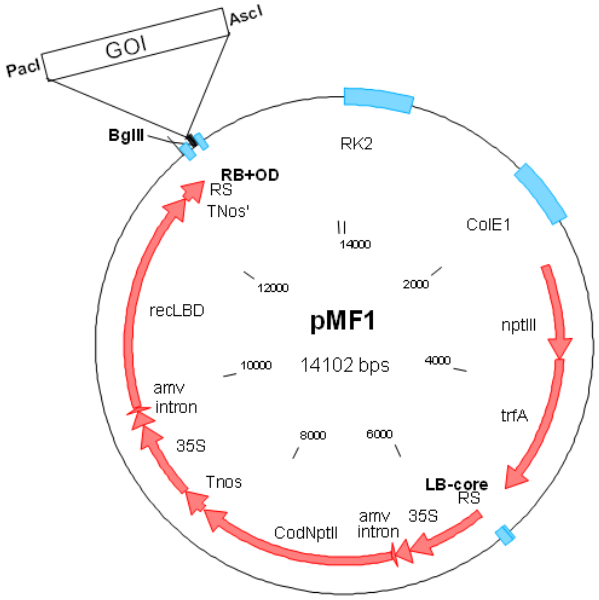

Figure 2. The Vector map of pMF1. The sites with **bold** letters *AscI* and *PacI* were used in cloning gene constructs into the binary vector pMF1 and *BglII* was used for digestion for Southern blotting, GOI represents gene of interest (Schaart et al. 2010a).

Plant transformation and regeneration

The *A. tumefaciens* strains containing the different constructs were cultured individually overnight in 100 ml LB medium with the appropriate antibiotics on a gyrorotary shaker at 28° C. After centrifugation of the culture (3000 rpm for 25 minutes) the pellet was resuspended in liquid MS medium supplemented with 3% (w/v) sucrose (pH 5.2) and diluted till an optical density of 0.4-0.8 was reached (Puite and Schaart 1996).

Four-week-old, *in vitro* propagated cv. 'Gala' shoots were used for plant transformation as described by Puite and Schaart (1996) with a slight modification. Leaves were used as explant source and for this, the top four leaves were taken and transferred to a sterile Petri dish filled with 15 ml *A. tumefaciens* suspension for inoculation. The leaves were cut into 0.5 cm^2 pieces and incubated for 30 minutes. For each construct on an average 275-300 leaf explants per experiment were subjected to inoculation with *Agrobacterium*. Then the explants were dried on a sterile filter paper and transferred for co-cultivation to shoot induction medium (SIM) consisting of MS medium (Murashige and Skoog, 1962) with vitamins, micro and macro elements, 3% (w/v) sorbitol, 9.9 µM thidiazuron (TDZ), 0.5 µM NAA, 13.3 µM BAP, 0.3% (w/v) Gelrite, pH 5.8 for 4 days. After 4 days the explants were transferred to regeneration/selection medium, i.e. SIM with antibiotics, 100 mg/l kanamycin for selection of transformants and 250 mg/l cefotaxim (to get rid of *A. tumefaciens*). Then, they were placed in the dark in a growth chamber at 24°C.

Subculturing was done every four weeks and explants were transferred to fresh SIM with antibiotics 100 mg/l kanamycin and 250 mg/l cefotaxim. After culturing in the dark for 12 weeks, callus had been produced on the explants and shoot-like structures started emerging from the calli. They were then exposed to diffused light and later gradually to full light conditions of 7000 lux (16 hrs) to avoid direct light stress (Espley et al. 2007). When the shoots were big enough, they were isolated and placed in shoot elongation medium (SEM) containing MS with vitamins, micro and macro elements, 1% (w/v) galactose, 2% (w/v) sucrose, 0.5 µM NAA, 4.4 µM BAP, 0.9% (w/v) Daishin agar, pH 5.8 for 4 weeks with 16h light/8hr dark at 24°C. When the shoots reached the stage where they looked like a complete, elongated plantlet, they were transferred to shoot propagation medium (SPM) containing MS with vitamins, micro and macro elements, 3% (w/v) sucrose, 96 mg/l FeEDDHA, 3.1 µM BAP, 0.9% (w/v/) Daishin agar, pH 5.8 for further multiplication of the clone. Putative transformants were clonally propagated for further analysis.

Molecular analysis of transformants

Genomic DNA from putative transformants was isolated as described for Diversity Arrays Technology (DArT) (Jaccoud et al 2001). All the putative transformants were analysed for the presence of inserted genes using *HcrVf1* and *HcrVf2* gene specific primers and with *nptIII,* and *trfA* primers for the presence of vector backbone (primers mentioned in Table 1). The PCR reaction included 10X Supertaq^{TM} buffer, 5mM dNTPs, 10µM each of forward and reverse primers, 0.5 U Supertaq^{TM} DNA polymerase making a total volume of 20 µl. The PCR conditions were as follows: 96° C for 5 minutes, followed by 30 cycles of 96°C for 30 seconds, 55°C for 45 seconds and 72°C for 1 minute and 30 seconds and final extension at 72°C for 10 minutes to generate an internal fragment of 505 bp for both *HcrVf1* and *HcrVf2*. Similar PCR conditions were used for *nptIII* and *trfA* to generate a fragment of 475 bp and 345 bp respectively.

Table. 1 Primers used in the cloning of *HcrVf* fragments and in molecular analysis of *HcrVf* transformants

Primers	Sequence	Fragment length (bp)
HcrVf1- Forward	5'-tctatctcagtagtttctataattcc-3'	505
HcrVf1-Reverse	5'-gtagttactctcaagattaagaactt-3'	
HcrVf2-Forward	5'-ctcaatctcagtagtttctatgga-3'	505
HcrVf2-Reverse	5'-cccccgagattaagagttg-3'	
SP*HcrVf1*-Forward	5'-***ggcgcgccgcgatcgc***gggtcttaaattccacacgta-3'	3840
SP*HcrVf1*-Reverse	5'-***acgcgt***tcacacatttctcttgtcattc-3'	
LP*HcrVf1*-Forward	5'-***ggcgcgccgcgatcgc***tctctcccaatttctttagggtta-3'	5537
LP*HcrVf1*-Reverse	5'-***acgcgt***ccattttcacacatttctcttgtc-3'	
SP*HcrVf2*-Forward	5'-***ggcgcgccgcgatcgc***ttccaagtggggtcttagattaac-3'	3668
SP*HcrVf2*-Reverse	5'-ttaattaaacgcgtaatccctaaaccattttcacacat-3'	
LP*HcrVf2*-Forward	5'-***ggcgcgccgcgatcgc***cgattcgttacaacagaagtgaac-3'	5390
LP*HcrVf2*-Reverse	5 –ttaattaaacgcgtatccctaaaccattttcacacatt-3'	
NPTII- Forward	5'-tcggctatgactgggcacaacaga-3'	721
NPTII- Reverse	5'-aagaaggcgatagaaggcgatgcg-3'	
TrfA-Froward	5'- cgaggaactatgacgacca- 3'	345
TrfA-Reverse	5'- ccacaccagttcgtcatcgt- 3'	
NPTIII-Forward	5'- catgatggctggagcaatct- 3'	475
NPTIII-Reverse	5'- agctcgacatactgttcttc- 3'	

Table 1. (continued). Primers used in quantitative RT-PCR

Primers	Gene Bank Accession	Sequence	Fragment length (bp)	Efficiency
HcrVf1- Forward	AY397723	5'- ctgtttaaccaaaaagaccttgcc -3'	117	1.90
HcrVf1-Reverse		5'- gtagttactctcaagattaagaactt -3'		
HcrVf2-Forward	AJ297740	5'- cttgatccgattcccaaattgt -3'	131	1.92
HcrVf2-Reverse		5'- cccccgagattaagagttg-3'		
MdActin-Forward	DT002474	5'-ctatgttccctggtattgcagacc-3'	82	1.96
MdActin-Reverse		5'-gccacaaccttgtttttcatgc-3'		

Bold and *italics* indicate *Asc*1 and *AsiS*1 restriction sites, **Bold**, *italics* and underline indicate *Mlu*1 restriction siteUnderline indicates *Pac*1 and *Mlu*1 restriction sites

T-DNA integration was analyzed by Southern hybridization, as described by Southern (1975) with slight modifications. Initially, 20 µg of genomic DNA was digested overnight at 37°C with the restriction enzyme *BglII*. The DNA fragments were separated on a 1% (w/v) agarose gel overnight with a very low voltage of 25V. DNA fragments were blotted onto a positively charged Hybond N+ nylon membrane (Amersham, little Chalfont, UK). The *nptII* probe was prepared using primers mentioned in the Table 1 to amplify a fragment of 721 bp. The *nptII* probe was mixed with "random oligomers" and radioactively labeled with 32^{P} d**C**TP according to the manufactures guidelines and use for hybridization. Untransformed cv. 'Gala' was used as negative control and plasmid containing the *nptII* gene was used as positive control.

Micrografting

The method of micrografting is essentially as described by Lane et al. (2003). The complete pictorial description of the micrografting procedure has been given in Figure 3.
Rootstock preparation: Initially, 210 1-year-old apple seedlings derived from a cross between Golden Delicious x Baskatong and 190 apple seedlings derived from a cross between Elstar x Baskatong obtained from the NAKTuinbouw (Horst, The Netherlands) were planted in pots filled with soil. After 1-2 weeks multiple buds were developed into branches with leaves. Only one of these branches was retained to get a good and healthy,

growing shoot. After approximately one month of growth, it was ready for grafting, acting as a rootstock. A horizontal cut was made at approximately 5-6 leaves from the base of the shoot where the stem had a diameter of approximately 0.5 cm and an incision was made to cut across the cambium like a letter 'V' making a wedge.

Scion preparation: The in vitro transgenic plantlets were used as the scions for grafting. At the lower part of the shoot the surface layer of cells was scraped off such that the cambium became exposed on two opposite sides of the scion. Subsequently, it was placed in a Petri dish containing water in order to avoid drying of the scion shoot.

Micro grafting: The prepared scion was inserted in the 'V' shaped wedge made in the rootstock and self-sealing latex bandage (The Sealtex, Maryland, USA) was used to cover the part of the rootstock and the scion union. Water was sprayed over the whole plant to moisten it thoroughly. Then, two bent, metal wires were put across each other and inserted into the pots; a polythene bag was placed as a cover over the metal wires to ensure maintenance of high humidity. Finally, a rubber band was placed tightly around the pot fixing the polythene bag. The height of the wires and bag was enough to allow the graft inside to stay without touching the bag.

Micrograft development: The air in the polythene bag was refreshed one week after micrografting. After two weeks small holes were made to the polythene bag in order to allow air exchange and exposure of the grafts gradually to lower humidity conditions. After 3 weeks the polythene bags were removed and the scions allowed to grow further. Four to six weeks after micrografting, the plants were ready for scab inoculations having developed at least four, new, young leaves. At this stage plant material was harvested for DNA and RNA extraction.

RNA isolation and Quantitative RT-PCR

Young leaves harvested from the plants that had been transferred to the greenhouse to perform the scab assay (described in detail in Chapter 4) were used to isolate total RNA using the RNeasy mini kit (Qiagen, Carlsbad, CA, USA). The RNA samples were run on a 1% (w/v) agarose gel to determine the RNA quality. An aliquot total RNA of 2µg was used to treat with *DNaseI* (Invitrogen Carlsbad, CA, USA) and subsequently reverse transcribed with a unique blend of oligo (dT) and random primers to synthesise cDNA using iScript first strand cDNA synthesis kit (Bio-rad Hercules, CA, USA). The reactions were performed according to the manufactures guidelines. Quantitative RT-PCR (qRT-PCR) was carried out to check the expression levels of the *HcrVf* genes and to correlate the expression with the gene copy number. Cv. 'Santana' is a natural resistant variety carrying both *HcrVf1* and

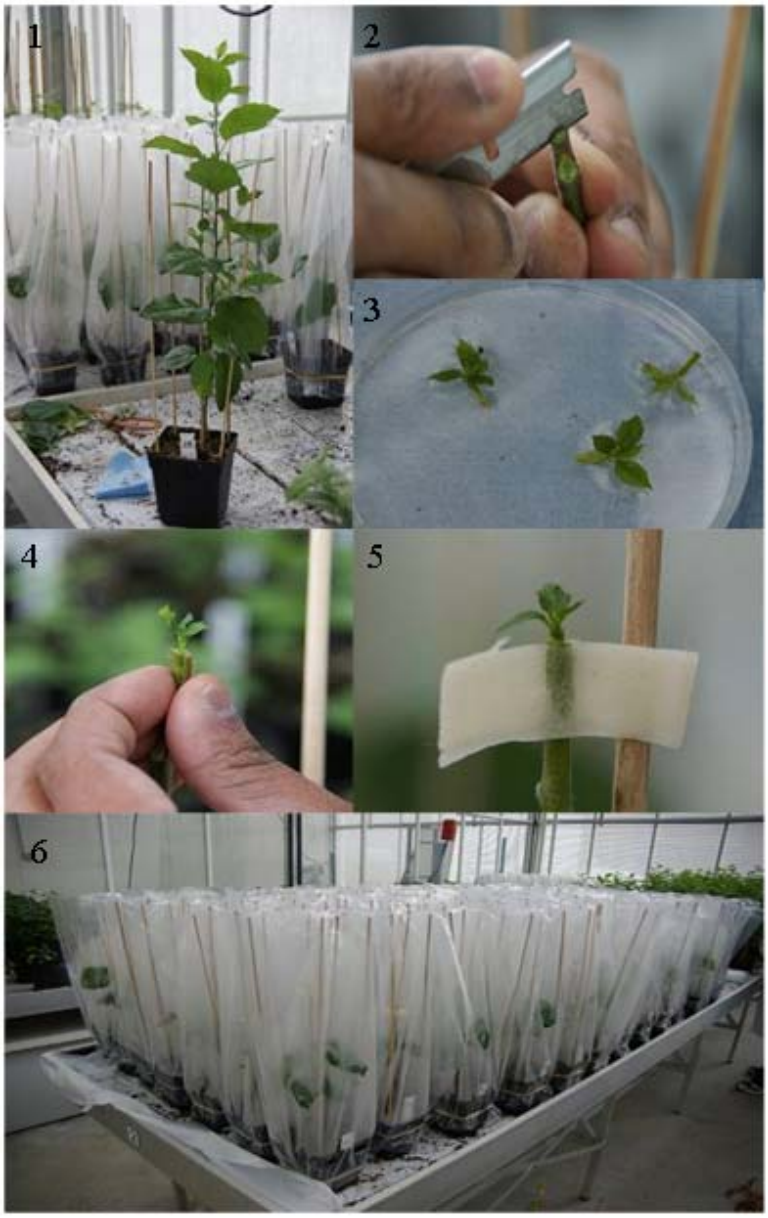

Figure 3. Sequential steps in Micrografting. 1: Apple rootstock before micrografting, 2: Making a "V" incision in the rootstock, 3: Prepared apple scions ready for micrografting, 4: Scion inserted in apple rootstock, 5: Sealtex tape around the graft union, 6: Micrografted plants with polythene bags on in the green house.

HcrVf2 genes. The expression of cisgenes/intragenes was measured as fold change by comparing with relative expression in cv. 'Santana'. Resistant control cv. 'Santana' was considered as one fold. Susceptible control "untransformed cv. 'Gala'" was assumed as zero due to the absence of *HcrVf* genes.

The qRT-PCR was performed with iQ SYBR® green super mix (Bio-rad) with MyiQ Single Color Real time detection system. As endogenous reference the β-actin gene (Accession number DT002474) was used. The primers used for qRT-PCR are presented in Table 1. Primer efficiencies were calculated through different dilution series of cDNA (Rebrikov and Trofimov 2006). All the PCR reactions were carried out in duplicates. *HcrVf* gene expression levels were analysed by using relative quantification method i.e. $2^{-\Delta\Delta Ct}$ method through qRT-PCR (Li et al. 2004). The threshold cycle (Ct) is the PCR cycle at which a statistically significant increase in the transcript is first detected.

The normalized Ct value difference (ΔCt) was calculated from Ct (*HcrVf*)-Ct (*Actin*) for all the reactions. The ΔCt value of cv. 'Santana' which has one copy of the natural *HcrVf* genes was chosen as reference sample and for all samples relative ΔΔCt values were calculated using ΔCt (reference sample- cv. 'Santana') – ΔCt (target sample). Finally the transgene expression levels were determined as fold change using the formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

The gene expression experiment was conducted to study the expressions of *HcrVf1* and *HcrVf2* genes under control of the short promoter, long promoter and rubisco promoter. The expression fold change is the experimental unit. The experimental design is unbalanced due to variation in number of replications. The data were analyzed through Student's t-test (unpaired) by comparing two promoters at a time.

Results

Gene amplification

Four independent BAC pools namely 12 and 199 for *HcrVf1*, 105 and 228 for *HcrVf2* were identified. The particular BAC clones harboring these genes were also isolated. The complete gene sequences including promoters of two different lengths, coding sequences and terminators as a single stretch for both genes were amplified (Figure 4) using the BAC clones as template. Next to this, two constructs with the P_{MdRbc} and T_{MdRbc} with both *HcrVf* genes were produced. The expected fragment sizes (boxed fragments in Figure 4)

were isolated for further processing. SP*HcrVf1* and LP*HcrVf1* were integrated at *AscI* site at the multiple cloning sites of the marker-free vector pMF1. SP*HcrVf2*, LP*HcrVf2* and P_{MdRbc}*HcrVf1*, P_{MdRbc}*HcVf2* were introduced at *AscI-PacI* and *PacI-AscI* sites respectively in pMF1 at the multiple cloning sites. The individual binary vectors were introduced into *Agrobacterium* strain AGL0 and after verification by PCR the strains were used for plant transformation.

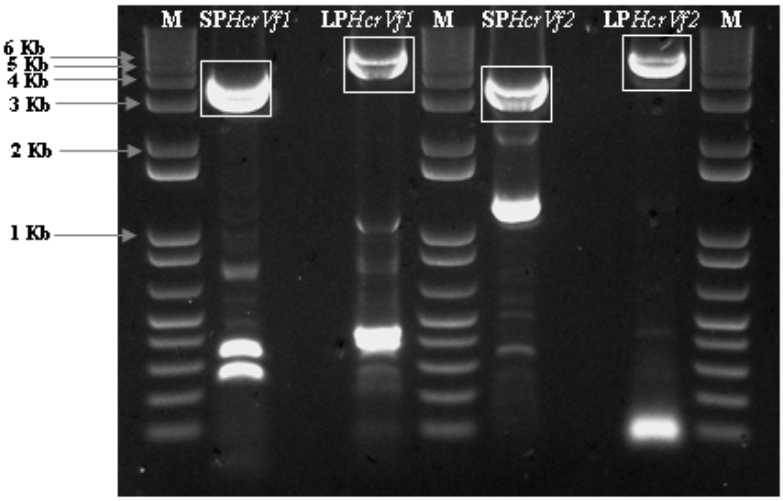

Figure 4 Amplification of full length *HcrVf* genes by PCR from BAC clones 12 (*HcrVf1*) and 105 (*HcrVf2*). M-1 Kb+ DNA ladder, SP*HcrVf1*- Short promoter *HcrVf1*, LP*HcrVf1*- Long promoter *HcrVf1,* SP*HcrVf2*- Short promoter *HcrVf2*, LP*HcrVf2*- Long promoter *HcrVf2*

Plant transformation and regeneration

For constructs SP*HcrVf*l 300, LP*HcrVf1* 300, P_{MdRbc}*HcrVf1* 275, SP*HcrVf2* 300, LP*HcrVf2* 200, P_{MdRbc}*HcrVf2* 300 explants resulted in ten, nine, ten, six, five, nine putative individual transgenic lines, respectively. The average transformation efficiency was in the range of 2-3%. After several subcultures, six independent transgenic lines were obtained for SP*HcrVf1*, LP*HcrVf1,* P_{MdRbc}*HcrVf1* and P_{MdRbc}*HcrVf2*. Four and three independent transgenic lines were obtained for SP*HcrVf2* and LP*HcrVf2* respectively.

Molecular analysis of transformants

All the *HcrVf1* and *HcrVf2* plant transformants were checked for the presence of the respective cisgenes using *HcrVf1* and *HcrVf2* specific primers through PCR and proved to be positive (data not shown).

T-DNA integration and copy number determination were analyzed by Southern hybridization (Table 2 and Figure 5). Out of 26 transformants used in the estimation of copy number of inserted gene 17 have a single T-DNA insert, seven were having two inserts, while two transformants could not be analyzed.

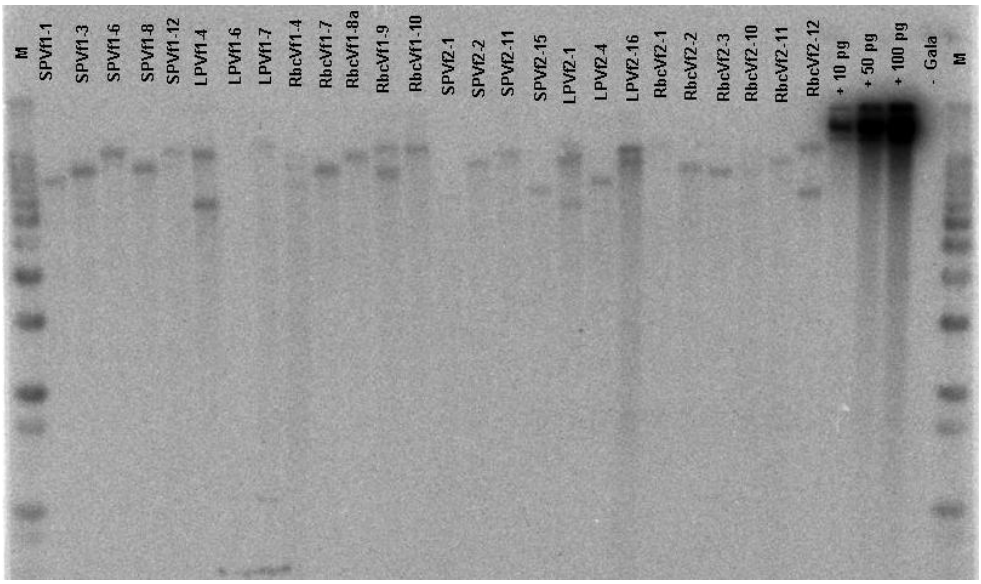

Figure 5. Estimation of transgene copy number apple transformants. Probe: *nptII*; digestion by *BglII*. M= 1kb+ DNA ladder, + = positive control (plasmid), - = negative control (untransformed 'Gala')

Quantitative RT-PCR

There was a wide variation in the expression of the *HcrVf* genes, under control of different promoters. The relative expression of the genes is given in the Table 2. The expressions of SP*HcrVf1*, LP*HcrVf1* and P_{MdRbc}*HcrVf1* were in the range of 0.4 to 2.3, 5.5 to 20.4, and 223 to 762 fold respectively in relation to the expression in cv. 'Santana'. The expressions of SP*HcrVf2*, LP*HcrVf2* and P_{MdRbc}*HcrVf2* were in the range of 0.23 to 1.2, 1.1 to 7.1, and 57 to 163 respectively in relation to the expression in cv. 'Santana'.

Analyzing the data statistically in case of *HcrVf1*, expression in plants transformed with LP*HcrVf1* was significantly higher than in plants transformed with SP*HcrVf1*, so the long promoter gave higher expression than the short promoter. The apple rubisco promoter performed significantly better than the long promoter in giving high expression levels (Figure 6). In case of *HcrVf2*, the SP*HcrVf2* and LP*HcrVf2* promoters' performance was not significantly different. Still, performance of P_{MdRbcS}*HcrVf2* was significantly higher than both SP*HcrVf2* and LP*HcrVf2* (Figure 7). Due to large variation in the expression between SP*HcrVf*, LP*HcrVf*, P_{MdRbc}*HcrVf* and for visual convenience two scales have been plotted together (Figures 6 and 7).

Table 2. The relative expression in *HcrVf* transformants compared to expression in cv. 'Santana' that contains the *HcrVf* genes because of classical breeding.

Transformants	Relative expression of *HcrVf1*	Inserted gene copy number
'Gala'	0.12	-
'Santana'	1.00	-
SP*HcrVf1*-1	0.43	1
SP*HcrVf1*-3	0.94	1
SP*HcrVf1*-6	1.35	1
SP*HcrVf1*-8	0.90	1
SP*HcrVf1*-11	0.79	ND
SP*HcrVf1*-12	2.4	1
LP*HcrVf1*-1	5.9	ND
LP*HcrVf1*-3	7.1	ND
LP*HcrVf1*-4	9.6	2
LP*HcrVf1*-6	5.5	ND
LP*HcrVf1*-7	13	1
LP*HcrVf1*-8	20	ND
P_{MdRbc}*HcrVf1*-4	762	2
P_{MdRbc}*HcrVf1*-7	238	1
P_{MdRbc}*HcrVf1*-8a	249	1
P_{MdRbc}*HcrVf1*-8b	223	ND
P_{MdRbc}*HcrVf1*-9	421	2
P_{MdRbc}*HcrVf1*-10	240	1

Table 2. (continued) The relative expression in *HcrVf* transformants compared to expression in cv 'Santana' that contains the *HcrVf* genes because of classical breeding.

Transformants	Relative expression of *HcrVf2*	Inserted gene copy number
'Gala'	0.00	-
'Santana'	1.00	-
SP*HcrVf2*-1	0.23	1
SP*HcrVf2*-2	0.30	1
SP*HcrVf2*-11	0.84	2
SP*HcrVf2*-15	1.2	1
LP*HcrVf2*-1	1.6	2
LP*HcrVf2*-4	7.1	1
LP*HcrVf2*-16	1.1	2
P_{MdRbc}*HcrVf2*-1	66	ND
P_{MdRbc}*HcrVf2*-2	57	1
P_{MdRbc}*HcrVf2*-3	81	1
P_{MdRbc}*HcrVf2*-10	121	1
P_{MdRbc}*HcrVf2*-11	78	1
P_{MdRbc}*HcrVf2*-12	163	2

ND-not determined

Discussion

Two of the four genes present within the *Vf* gene cluster, i.e. *HcrVf1* and *HcrVf2* were checked for the effect of different native and non-native regulatory elements on the expression. The length of native promoters and the nature of the promoter, i.e. the small subunit rubisco promoter, were varied in order to determine whether cis-elements in the 5' upstream promoter region to the genes were important factors in influencing expression levels and to determine whether higher expression levels would result in higher resistance levels (see Chapter 4). This study is aimed at the finding out whether longer regulatory

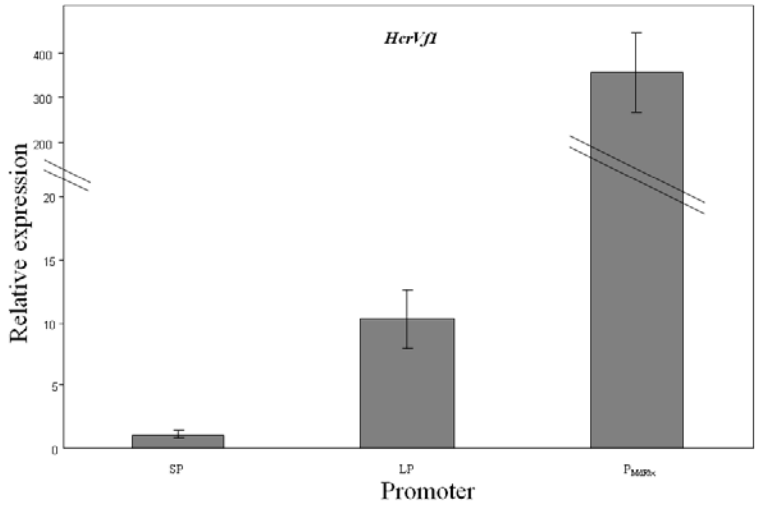

Figure 6. Relative expression of *HcrVf1* as observed under control of different gene promoters. SP = short promoter; LP = long promoter; P_{MdRbc} = apple rubisco promoter. Santana as reference was set at 1 (see also Table 2). For visual convenience two scales have been plotted together. Two oblique lines indicate change in the scale in Y axis.

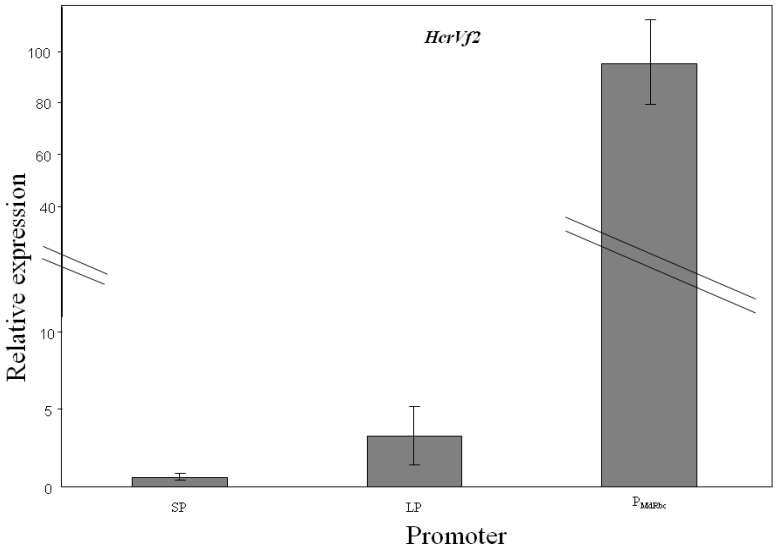

Figure 7. Relative expression of *HcrVf2* as observed under control of different gene promoters. SP = short promoter; LP = long promoter; P_{MdRbc} = apple rubisco promoter. Santana as reference was set at 1 (see also Table 2). For visual convenience two scales have been plotted together. Two oblique lines indicate change in the scale in Y axis.

elements would influence either positively or negatively the expression of the gene compared to shorter regulatory elements and whether a correlation between the inserted gene copy number and gene expression existed. Due to difficulty in cloning of *HcrVf2* gene with SP and LP only four and three apple transformants were obtained for SP*HcrVf2* and LP*HcrVf2* respectively. Twenty-six apple transformants were analyzed for transgene copy number through Southern hybridization. Seventeen of them were found to have a single copy inserted and seven carried two T-DNA inserts. Both types of transformants showed a wide variation in transgene expression and neither positive nor negative correlation could be found between copy number and expression of the genes.

Expression of *HcrVf* genes

In *HcrVf1* transformants, the relative expression of LP*HcrVf1* and SP*HcrVf1* is significantly different. Expression of LP*HcrVf1*, so the long promoter of 1996 bp, was on average ten times more than that of SP*HcrVf1*, representing the short promoter of 312 bp (Table 3). This could be an indication for the presence of 5'upstream cis-acting elements having a positive effect on driving expression of *HcrVf1*. Similar results have been obtained in other crops, e.g. in kidney bean (*Phaseolus vulgaris* L.), where seed-specific Unknown Seed Protein (USP) promoters, short (637 bp) and long (1149 bp) were used to direct reporter gene expression (Zakharov et al. 2004). They showed that the long promoter was three times stronger in expression than the short promoter. In a similar study, expression of reporter gene *gus* in tobacco was studied with a series of deleted promoter fragments (Bäumelein et al. 1991). They showed that major expression-enhancing cis-elements are present beyond 200 bp upstream from the transcription start site. In apple, Silfverberg-Dilworth et al. (2005) studied different *Vf*-promoter fragments by combining them with the *gus* reporter gene and they observed results that seem to be in contrast to the findings reported here. They found that an *HcrVf1*-derived promoter with a length of 1200 bp showed lower *gus* expression than shorter *HcrVf1* promoters. They concluded that use of promoter fragments greater than 1 kb should be avoided because of reduced activity in tobacco in their experiment. Similar results were obtained when they combined the *gus* reporter gene with different lengths of *HcrVf2* promoter and showed that *HcrVf2* with a promoter length of 779 bp showed less expression compared to the gene controlled by a short promoter of 288 bp.

In our study, the expression of LP*HcrVf2* and SP*HcrVf2* proved not to be significantly different, even though expression of LP*HcrVf2* was on an average five times more than that of SP*HcrVf2* (Table 3). Szankowski et al. (2009) found that the *HcrVf2* gene

with a native promoter of 779 bp was expressed twice as much as compared to a short promoter of 288 bp. All these data indicated that long promoters provide better expression than short promoters (see also Lee et al. 2000).

Considerable variation was observed in the expression of SP*HcrVf2* when we compare our results with that of Szankowski et al. (2009). Our results show expression levels comparable to cv. 'Santana', while they found expression to be 50-100 times higher than their resistant control cultivar cv. 'Florina'. This could be attributed to the resistant cultivar used as reference but also to the use of a native terminator sequence by us and the *nos* terminator sequence by Szankowski et al. (2009). The significance of terminator sequences in directing expression was first reported by Dean et al. (1989) and confirmed by Ingelbrecht et al. (1989). Schaart et al. (2010b) demonstrated in *gus* expression studies in tobacco that the apple rubisco terminator gave significantly higher expression than the *nos* terminator in combinations with both the apple rubisco promoter as well as with the *35S* promoter.

Correlation between Inserted copy number and gene expression

In our experiments no correlation was observed between inserted gene copy and gene expression. Correlation studies were also performed earlier by many researchers and no clear cut correlations were found between the copy number of transgene and level of expression. (Jones et al. 1985; Zanek et al. 2009). Zeng et al. (2009) studied the integration of fused *bgt* gene (consisting of the insecticidal toxin gene from the spider (*Atrax robustus*) and the C terminal of *Cry IA (b)* gene from *Bacillus thuringiensis*) in transgenic birch and showed no significant correlation between copy number and expression level of the *bgt* gene.

On the other hand in another study Min et al (2009) described negative correlation when they observed variation in the expression levels of transgenes in T1 tobacco plants carrying multiple T-DNA copies. They studied two transgenic tobacco lines, line 26 with single copy and line 28 with three copies and showed in line 28 T-DNA were arranged in head-to-head and tail-to-tail repeats at the same locus. They suggested that multicopy transgenes cause silencing of the transgenes by means of cosuppression. In a study in Citrus, it was found that there was a significant negative correlation between transgene copy number and *gus* activity (Cervera et al. 2000).

Most variation in the observed expression among different lines (transformation events) with the same number of copies is thought to be due to the position effect of the inserted T-DNA (Dean et al. 1988) E.g. from two of the LP*HcrVf1* transformants, LP*HcrVf1*-7 and LP*HcrVf1*-4, LP*HcrVf1*-7 has a single copy insert and showed a four fold higher gene expression level than LP*HcrVf1*-4 which has two transgene copies. In the case of two P_{MdRbc}*HcrVf2* transformants, P_{MdRbc}*HcrVf2*-11 and P_{MdRbc}*HcrVf2*-12, P_{MdRbc}*HcrVf2*-11 has

one transgene copy and showed more than two fold lower gene expression than P_{MdRbc}*HcrVf2*-12 which has two transgene copies. The fact that no correlation existed between the inserted copy number and gene expression may be due to the position effect. Insertion of T-DNA could have occurred at a position close to where expression enhancers such as Matrix Attachment Regions (MARs) are present. MARs are involved in determining position effects (Girod et al. 2007). They are DNA sequences that bind specifically to a network of proteinaceous fibres, called nuclear matrix, which permeates the nucleus (Allen et al. 2004). The transgene expression will be higher and more stable when it is integrated next to these kinds of MARs (Allen et al. 2000). Therefore, position effect may play very important role in the transgene expression.

Low expression of inserted genes in *HcrVf* transformants may also be due to methylation which may lead to inactivation of transgenes (Kilby et al. 1992). They described that loss of resistance to kanamycin was associated with methylation of an *SstII* site in the *nos* promoter and that upon treatment with the demethylation agent 5-azacytidine the resistance was restored. Weber et al. (1990) studied methylation effects in gene expression. They transferred a hemimethylated chimeric gene with a *CaMV35S* promoter, *β-glucuronidase* coding region and the *nos* terminator into protoplasts of tobacco through polyethylene glycol mediated transfection. The integrated transgene was constitutively hypermethylated at CpG and CpNpG and this led to inactivation of the *β-glucuronidase* gene. In another study, the nature of the T-DNA insertion region was studied in Arabidopsis (Mirza 2005) by transforming the *CH-42* gene using kanamycin resistance gene for selection. They identified two independent transgenic lines where the *CH-42* gene was inactive. Methylation was observed in both inactive *CH-42* lines through Southern blots which were produced by digesting genomic DNA with methylation-sensitive restriction enzymes *MspI/HpaII*.

Gene pyramiding is a concept to have a broad spectrum of resistance against a pathogen. One way of gene pyramiding is through stacking of *HcrVf1* and *HcrVf2* genes together in one binary construct. Senthilkumar et al. (2010) developed dual resistant transgenic tobacco against insect and phytopathogens using *Spormin* (trypsin inhibitor) from sweet potato and *CeCPI* (phytocystatin) from taro by stacking both the genes together in a binary vector. We prepared construct LP*HcrVf1*+LP*HcrVf2* and transformed to cv. 'Gala'. During regeneration either explants failed to produce calli or if produced became brown and explants failed to survive. One hypothesis could be that due to the high expression of both the genes, the resulting gene products reached levels where they became toxic and this led to lethality.

Conclusion

In conclusion *HcrVf1* under the regulation of long promoters was expressed at higher levels than by short promoters and this difference proved statistically significant. However, there was no statistically significant difference between short and long promoters in case of *HcrVf2*. More transformants are needed to make a conclusive statement. Highest expression of *HcrVf1* and *HcrVf2* was observed when expressed under the regulation of the apple rubisco promoter. This result is in line with the result obtained by Schaart et al. (2010b). Most of the apple transformants (65%) have a single T-DNA insert. Among *HcrVf* transformants no correlation was observed between the inserted gene copy number and gene expression.

Acknowledgements

We thank Ms. Iris Capel and Ms. Linda Kodde (Wageningen UR Plant Breeding, The Netherlands) for their help during the lab experiments. Dr. Chris Maliepard (Wageningen UR Plant Breeding, Wageningen, The Netherlands) for all the help and advice during the statistical analysis of the results.

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Chapter 4

Resistance evaluation and expression analysis of *HcrVf* transformants

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Abstract

Present day apple varieties are susceptible to apple scab which is caused by the fungus *Venturia inaequalis.* Apple transformants with *HcrVf1* or *HcrVf2* were generated. These genes were under control of their native regulatory elements (cisgenic) in two lengths, i.e. *HcrVf1* short promoter (SP)-312 bp, *HcrVf1* long promoter (LP)-1990 bp; *HcrVf2* short promoter (SP)-288 bp, *HcrVf2* long promoter (LP)-2000 bp. As a control, the apple rubisco promoter (intragenic) (P_{MdRbc} -1600 bp) which generally yields high expression levels, was also used to express *HcrVf1* and *HcrVf2*. The high quality cultivar 'Gala' was used for plant transformation. In vitro propagated shoots from individual plant transformants were micrografted onto apple seedling rootstocks to enable scab disease tests. Scab tests were conducted in a temperature and humidity controlled greenhouse. From six replicates of micrografted plants for each transgenic line (event) the top four young, expanding leaves were used for inoculation with monoconidial isolate EU-B05 ($5x10^5$ conidia/ml) which belongs to race 1 of *V. inaequalis*. Plants were scored for sporulation 17 days after inoculation. Plant transformants were tested for expression of *HcrVf* genes through quantitative RT-PCR (qRT-PCR) and expression was linked to the observed resistance level. All the *HcrVf1* transformants (LP, SP and P_{MdRbc} driven) showed sporulation to a similar extent as untransformed 'Gala'. On the other hand 10 out of 13 *HcrVf2* transformants were found to be resistant to a level similar as cv. 'Santana', a resistant cultivar that has obtained *HcrVf1* and *HcrVf2* by conventional breeding. Higher expression levels for *HcrVf1* were observed than for *HcrVf2*, but without any resistance. Both P_{MdRbc}*HcrVf1* and P_{MdRbc}*HcrVf2* showed a very high expression level (respectively 300 and 100 times that of 'Santana'), but despite these high expression levels, P_{MdRbc}*HcrVf1* transformants showed complete fungal sporulation similar to untransformed 'Gala'. Consequently, it was concluded that only *HcrVf2* is functional in providing resistance to the scab fungal isolate EU-B05. The rubisco promoter combined with the *HcrVf2* gene gave the highest expression and resistance. With its native promoters, SP or LP, *HcrVf2* showed to be statistically similar in gene expression at levels comparable to the one observed in cv. 'Santana'. The best performing transformants with cisgenic and intragenic resistance genes have been selected for induced marker removal after recombination, the next step in making them marker-free.

Introduction

Present day apple varieties are susceptible to apple scab which is caused by the fungus *Venturia inaequalis. HcrVf* is until now the only isolated and functionally analyzed apple scab resistance gene (Vinatzer et al. 2001; Xu and Korban 2002). *HcrVf* is a gene cluster consisting of four paralogs *HcrVf1*, *HcrVf2*, *HcrVf3* and *HcrVf4* which are highly homologous to the *Cladosporium fulvum* resistance gene of tomato (Vinatzer et al. 2001). *HcrVf1*, *HcrVf2* and *HcrVf4* are complete genes and *HcrVf3* is thought to be a truncated gene with premature termination of transcription (Xu and Korban 2002). *HcrVf2* was predicted to code for an extracellular receptor, which protein should be present continuously (Szankowski et al. 2009).

Promoter lengths can determine the expression of genes. Silfverberg-Dilworth et al. (2005) studied different lengths of promoters for the genes *HcrVf1*, *HcrVf2* and *HcrVf4* by transcriptionally fusing them to an intron-containing *β–glucuronidase* (GUS). They concluded that 312 bp or 632 bp for *HcrVf1*, 288 bp for *HcrVf2* and 332 bp for *HcrVf4* were promoter lengths giving sufficiently high expression to use with the respective genes in genetic modification. The maximum length that they tested was 1574 bp and from literature it is known that regulatory cis-elements can be located as far as 2267 bp upstream (Chen et al. 2007). The resistance and expression of *HcrVf2* gene with native promoters with lengths of 115 bp, 288 bp, and 779 bp were studied (Szankowski et al. 2009). They observed that the *HcrVf2* gene with the 115 bp promoter showed sporulation after scab inoculation whereas *HcrVf2* with 288 bp and 779 bp promoters showed high resistance. The expression of *HcrVf2* with 288 bp and 779 bp promoters and the *nos* terminator in transgenic cv. 'Gala' lines was about 108 and 115 fold respectively, compared to cv. 'Florina', which is a cultivar that contains *HcrVf2* thanks to conventional breeding.

On the other hand, Malnoy et al. (2008) found that transformed lines of apple with *HcrVf1* and *HcrVf2* both showed enhanced resistance. Transformed lines carrying the *HcrVf4* gene were more susceptible than the controls (non-transformed Galaxy or McIntosh) following inoculation with the pathogen *V. inaequalis*. They observed an increase of 5 to 10 fold and 2 to 8 fold for *HcrVf1* transgene expression in Galaxy and McIntosh transformed lines respectively compared to control plants (Galaxy and McIntosh untransformed which have no *Vf* genes). Similarly *HcrVf2* transgene expression in Galaxy and McIntosh

transformed lines was increased 4 to 30 fold and 5 to 8 fold, respectively compared to the controls (Galaxy and McIntosh untransformed which have no *Vf* genes).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is an abundant protein in plants (Ellis 1979) and rubisco protein contents were correlated with total rubisco small subunit (RbcS) mRNA levels in rice (Suzuki et al. 2009). Using the promoter and terminator of the rubisco small subunit (*RbcS1*) of chrysanthemum, expression levels of 7 to 8 fold that provided by the constitutively expressed *CaMV35S* promoter were obtained (Outchkourov et al. 2003). The combination of the apple rubisco promoter and terminator (P_{MdRbc}) with the *gus* reporter gene resulted in similar expression levels compared to the *CaMV35S* promoter when expressed in tobacco (Schaart et al. 2010b).

In this study we report on the individual roles of *HcrVf1* and *HcrVf2* in conferring resistance to scab, and on the effect of different lengths of the native promoters for regulating expression. In addition, the effect of high expression levels using the rubisco promoter and terminator from apple was studied. The correlation between the levels of expression and the levels of resistance was investigated.

Materials and Methods

Plant transformation and regeneration

Two genes, *HcrVf1* and *HcrVf2*, were cloned with their native regulatory elements, i.e. for *HcrVf1* a short promoter (SP) of 312 bp length and a long promoter (LP) of 1990 bp, in both cases with the same terminator of 480 bp; for *HcrVf2* a short promoter (SP)-288 bp and a long promoter (LP)-2000 bp, both with the same terminator of 437 bp. The highly active apple rubisco promoter (P_{MdRbc}) of 1600 bp was also used in combination with, a terminator sequence of 593 bp from apple (T_{MdRbc}) to express *HcrVf1* and *HcrVf2.* Cultivar 'Gala' was used for plant transformation by *A. tumefaciens* strain AGL0 carrying the different constructs. Development of individual plant transformants carrying *HcrVf1* and *HcrVf2* genes and the molecular analysis of the transgenic lines representing individual transformation events are given in detail in the Materials and Methods part of Chapter 3.

Micrografting

The procedure of micrografting of apple transformants on to the apple rootstocks has been described in the Materials and Methods part of Chapter 3. Four to six weeks after

micrografting plants were ready for scab resistance assays. Also at this stage, plant material was harvested for DNA and RNA extraction as it was described in Chapter 3.

Scab resistance evaluation

Scab disease tests were conducted in a temperature and humidity controlled greenhouse. Tunnels using polythene plastic and a PVC framework were prepared to fit the tables within the greenhouse compartment and to be able to contain 198 plants. A conidial suspension of *V. inaequalis* isolate EU-B05 (Bus et al. 2005a) containing $5x10^5$ conidia/ml was prepared. The suspension was checked for germination in vitro and used if a germination rate of more than 90% was observed.

The top four young, expanding leaves of the scions were used for inoculation with the well–characterized, monoconidial isolate EU-B05. For each transgenic event six replicates were used unless stated otherwise. Inoculated plants were kept in the tunnel in the dark for 48 hours at 20°C and 100% relative humidity. The humidity in the tunnel was maintained by two vaporizers connected to a continuous water supply set at vaporization bursts with intervals of six min “off” and one min “on”. After those 48 hours, the plants were transferred outside the tunnel, but still kept in the same greenhouse compartment with the temperature set at 19°C during day and 16°C during night, day length of 16hr and relative humidity of 85%.

Disease symptoms were assessed macroscopically 17 days post inoculation and classified in eight classes as reported by Durel et al. (2003), indicative for the amount of sporulation as follows: class 0, 0% sporulation; class 1, 1–2% sporulation; class 2, 2–5% sporulation; class 3, 5–10% sporulation; class 4, 10–25% sporulation; class 5, 25–50% sporulation; class 6, 50–75% sporulation; class 7, 75–100% sporulation. This scale was adapted from Croxall et al. (1952).

RNA isolation and quantitative RT-PCR (qRT-PCR)

Young leaves were harvested from the plants that had been transferred to the greenhouse to perform scab test. The procedure of RNA isolation and qRT-PCR has been described in the Materials and Methods part of Chapter 3.

Statistical analysis

The scab disease experiment was conducted with Randomized Complete Block Design (RCBD). Micrografted plants were the experimental units. As a rule, six replicates were used for each transformation event; for each construct (Chapter 3) 3 to 6 independent lines were taken. In total, 31 events and 198 plants were investigated. All the statistical

analyses were performed using Genstat® 11 (Genstat® 2008). Since the treatments were not balanced, an ANOVA could not be used for analysis. In stead we used the linear mixed model (LMM) procedure.

The expression data were log transformed and correlation was studied between phenotypic data and log transformed expression data using Spearman's correlation.

Results

Scab resistance evaluation

All the 18 *HcrVf1* transformants, i.e. six independent transgenic lines of each SP*HcrVf1*, LP*HcrVf1*, and P_{MdRbc}*HcrVf1,* showed heavy sporulation. Sporulation was statistically similar to the level found on 'Gala' (susceptible control) which is shown in Figure 1. The high sporulation levels observed were independent of promoter type or promoter length. In some transgenic lines the sporulation was even more than in cv. 'Gala'.

On the other hand, 10 out of 13 *HcrVf2* transformants showed less or no sporulation and were statistically similar to cv. 'Santana' (resistant control) which is depicted in the Figure 2. All six independent transgenic lines of P_{MdRbc}*HcrVf2* showed no sporulation. Among the plants carrying the constructs with the native promoters, three out of four independent transgenic lines of SP*HcrVf2* showed less or no sporulation. Only one of the three independent transgenic lines of LP*HcrVf2* showed less or no sporulation. A view of the symptoms of sporulation as an indication of susceptibility and resistance offered by the respective promoters and genes used in this study is given in Figure 3.

Typically, *HcrVf* should give chlorotic spots as symptoms for a resistant reaction. Cv. 'Santana' is a representative of a resistant cultivar obtained by breeding and carries the *HcrVf* gene cluster and here, indeed chlorosis was observed. Also in SP*HcrVf2* transformants, similar chlorotic symptoms were observed. In the other two types of *HcrVf2* transformants this phenomenon was not observed or to a much lesser extent.

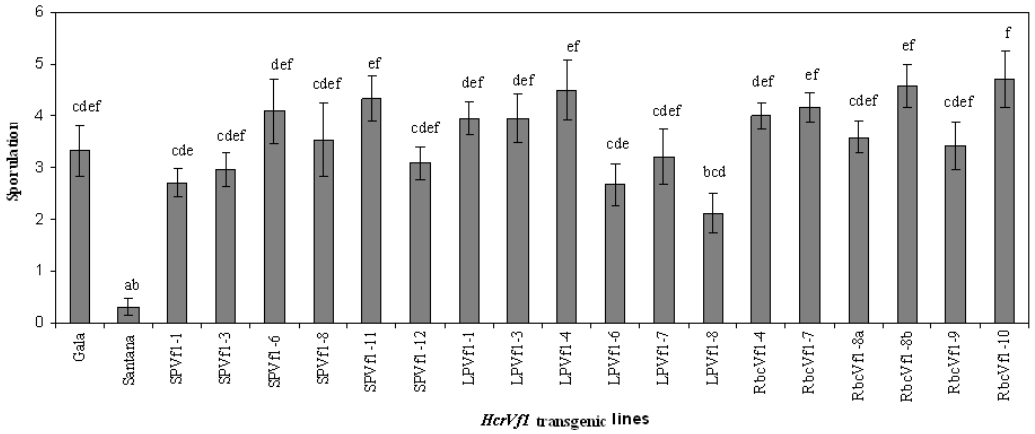

Figure 1. Sporulation of the *Vf* avirulent monoconidial isolate EU-B05 of *V. inaequalis* on the leaves of *HcrVf1* transgenic lines. Cvs. 'Santana' and 'Gala' were used as resistant control and susceptible control respectively. *Vf1*-*HcrVf1*, SP-short promoter, LP-long promoter, Rbc-apple rubisco promoter. The error bars represent the standard error of the mean.

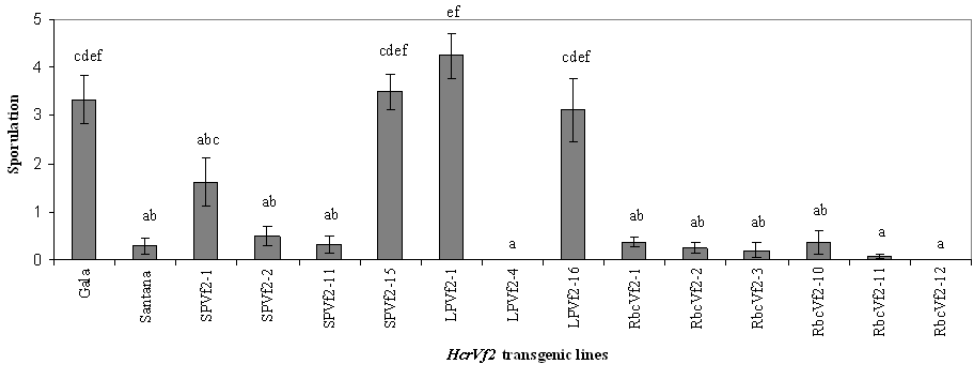

Figure 2. Sporulation of the *Vf* avirulent monoconidial isolate EU-B05 of *V. inaequalis* on the leaves of *HcrVf1* transgenic lines. Cvs. 'Santana' and 'Gala' were used as resistant control and susceptible control respectively. *Vf2*-*HcrVf2*, SP-short promoter, LP-long promoter, Rbc-apple rubisco promoter. The error bars represent the standard error of the mean.

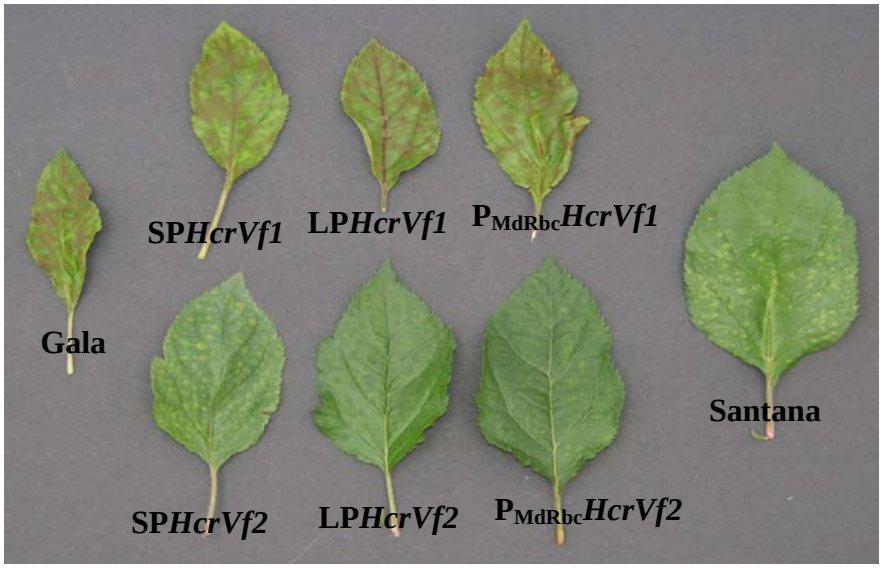

Figure 3. Sporulation of *Vf* avirulent monoconidial isolate EU-B05 of *V. inaequalis* as observed on leaves of *HcrVf* transgenic lines and on cv. 'Santana'(resistant control) and on cv. 'Gala' (susceptible control).

Quantitative RT-PCR (qRT-PCR)

In the gene expression study of the *HcrVf1* transformants, the short promoter *HcrVf1* provided in general lower expression, ranging from 0.4 to 2.3 fold, compared to the long promoter *HcrVf1* transgenic lines which were in the range of 5.5 to 20.4 fold in relation to the expression in cv. 'Santana'. This difference was statistically significant. The highest expression of the *HcrVf1* gene was obtained with the rubisco promoter, ranging from 223 to 762 fold in relation to the expression in cv. 'Santana'. The expression of *HcrVf* genes in plant transformants is given in Table 1. The relative expression levels of *HcrVf* genes are shown in Table 2.

Table 1. The Expression of *HcrVf* transformants in relation to cv. 'Santana' that contains the *HcrVf* genes because of classical breeding

Transformants	Relative expression of *HcrVf1*
'Gala'	0.12
'Santana'	1.00
SP*HcrVf1*-1	0.43
SP*HcrVf1*-3	0.94
SP*HcrVf1*-6	1.35
SP*HcrVf1*-8	0.90
SP*HcrVf1*-11	0.79
SP*HcrVf1*-12	2.4
LP*HcrVf1*-1	5.9
LP*HcrVf1*-3	7.1
LP*HcrVf1*-4	9.6
LP*HcrVf1*-6	5.5
LP*HcrVf1*-7	13
LP*HcrVf1*-8	20
P_{MdRbc}*HcrVf1*-4	762
P_{MdRbc}*HcrVf1*-7	238
P_{MdRbc}*HcrVf1*-8a	249
P_{MdRbc}*HcrVf1*-8b	223
P_{MdRbc}*HcrVf1*-9	421
P_{MdRbc}*HcrVf1*-10	240

Transformants	Relative expression of *HcrVf2*
'Gala'	0.00
'Santana'	1.00
SP*HcrVf2*-1	0.23
SP*HcrVf2*-2	0.30
SP*HcrVf2*-11	0.84
SP*HcrVf2*-15	1.2
LP*HcrVf2*-1	1.6
LP*HcrVf2*-4	7.1
LP*HcrVf2*-16	1.1
P_{MdRbc}*HcrVf2*-1	66
P_{MdRbc}*HcrVf2*-2	57
P_{MdRbc}*HcrVf2*-3	81
P_{MdRbc}*HcrVf2*-10	121
P_{MdRbc}*HcrVf2*-11	78
P_{MdRbc}*HcrVf2*-12	163

In the gene expression study of *HcrVf2* transformants, four short promoter *HcrVf2* transgenic lines and three long promoter transgenic lines showed similar expression levels. The observed expression levels ranged from 0.23 to 1.2 and 1.1 to 7.1 fold for SP*HcrVf2* and LP*HcrVf2* transgenic lines, respectively, again related to the expression of cv. 'Santana'. There was no significant difference between SP*HcrVf2* and LP*HcrVf2*. The six rubisco promoter *HcrVf2* transgenic lines showed the highest expression levels, which ranged from 57 to 163 fold in relation to the expression of cv. 'Santana'.

Table 2. Mean expression of the *HcrVf1* and *HcrVf2* genes regulated by the different promoters.

Gene	Promoter	Mean ± SD	SE
	'Santana'	1.00	
	'Gala'	0.00	
HcrVf1	SP	1.1 ± 0.7	0.3
	LP	10 ± 5.8	2.3
	P_{MdRbc}	355 ± 212	87
HcrVf2	SP	0.6 ± 0.4	0.2
	LP	3.3 ± 3.3	1.9
	P_{MdRbc}	94 ± 40	16

Cv. 'Santana'- resistant control, cv. 'Gala'- susceptible control, SP- short promoter, LP- long promoter, P_{MdRbc} - apple rubisco promoter, Mean of SP*HcrVf1,* LP*HcrVf1*, P_{MdRbc}*HcrVf1*, P_{MdRbc}*HcrVf2* is the average of six replication, mean of SP*HcrVf2* is the average of four replications, mean of LP*HcrVf2* is the average of three replications. ±SD represents standard deviation of the mean, SE represents the standard error of mean

Correlation between sporulation and gene expression

The sporulation data obtained from the greenhouse experiment and the gene expression data from qRT-PCR were used to study their correlation. In *HcrVf2* transformants a negative correlation was observed i.e. the higher the expression, the lower is the sporulation. The correlation co-efficient observed was -0.57 (P=0.007) between the expression and sporulation. All the P_{MdRbc}*HcrVf2* transformants were statistically similar to cv. 'Santana' in resistance and were highest in the gene expression. One of the LP*HcrVf2* transformants, LP*HcrVf2-4*, was the highest in expression among the native promoters, and still proved to be statistically similar to cv. 'Santana' in resistance. Two SP*HcrVf2* transformants, SP*HcrVf2*-2, SP*HcrVf2*-11 were statistically similar to cv. 'Santana' in resistance and were close to the level of cv. 'Santana' in the expression (0.30 and 0.84 times cv. 'Santana'). On the other hand SP*HcrVf2*-1, SP*HcrVf2*-15, LP*HcrVf2*-1, and LP*HcrVf2*-16 that also have expression levels close to cv. 'Santana', showed complete sporulation like untransformed, susceptible cv. 'Gala'. All the P_{MdRbc}*HcrVf2* transformants were statistically

similar to cv. 'Santana' in resistance and were the highest in gene expression (57 to 163 times cv. 'Santana').

Discussion

The apple scab resistance genes, *HcrVf1* and *HcrVf2*, with two promoter types (native and rubisco) and two native promoter lengths (short and long) were isolated or constructed and subsequently cloned into the marker-free pMF1 vector (Chapter 3). These gene-promoter combinations were transferred to the susceptible cultivar 'Gala' by *A. tumefaciens-mediated* transformation to study the resistance to scab isolate EU-B05 which belongs to race 1 of *V. inaequalis*. A correlation between the sporulation and the expression of the transgenes was investigated.

Micrografting

Micrografting proved to be a good option for taking the transformants to the greenhouse early in development. Root induction of in vitro plantlets or transformants generally takes 4-5 weeks which is about the same as the micrografting period. However, the efficiency of in vitro root induction in apple was found to be rather low, hampering successful transfer to the soil and the greenhouse for further testing (Puite and Schaart, 1996), whereas with micrografting the success rate for survival after transfer to the greenhouse can be as high as 100% (Lane et al. 2003). Six transformants from each of SP*HcrVf1*, LP*HcrVf1,* P_{MdRbc}*HcrVf1,* P_{MdRbc}*HcrVf2*, four transformants from SP*HcrVf2*, and three transformants from LP*HcrVf2* were micrografted onto apple seedling rootstocks with six replications each. After five weeks, the success rate in our hands was found to be 100 % and substantial growth of the grafted scions was observed with more than four new, young leaves being present to enable inoculations. The transformants were inoculated with EU-B05 which belongs to race 1 of *V. inaequalis*. Inoculated plants were scored after 17 days. Cultivar 'Santana' was used as resistant control and cultivar 'Gala' as susceptible control.

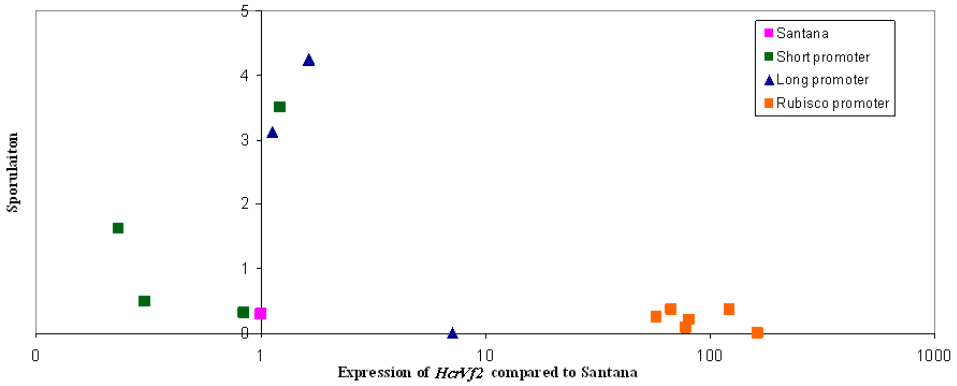

Figure 4. Correlation between sporulation of *Vf* avirulent monoconidial isolate EU-B05 of *V. inaequalis* and expression of the *HcrVf2* gene in apple transformants. X axis is displayed in logarithmic scale. 'Santana'- resistant control.

Scab resistance evaluation

In our experiment, all the *HcrVf1* transformants, irrespective of the type or length of promoter used, showed sporulation at levels similar to cv. 'Gala' resulting in complete susceptibility. This result was in contrast to the result obtained by Malnoy et al. (2008). They inserted *Vfa1* (which is the same as *HcrVf1*) and *Vfa2* (which is the same as *HcrVf2)*, under the control of their native promoters of about 2Kb in length individually in two susceptible cultivars namely 'Galaxy' and 'McIntosh'. They showed that *Vfa1* gave partial resistance to a mixed inoculum containing isolates 1805-2, 1777-8, 1771-2, 1778-6, and 1810-1 (representing five races [1 to 5] of *V. inaequalis*). They observed reduction in susceptibility to apple scab by 50% for *HcrVf1.* They hypothesized that this partial resistance could be due to the high concentration of inoculum (2.7×10^7 conidia/ml) during the scab disease assay, or to a mutation in *Vfa1* during different steps of the transformation process, or that the physical separation of *Vfa1* from the *Vf* cluster in their transformants, compared to the natural situation, might have resulted in lower levels of resistance.

In our experiment with *HcrVf2* transformants 10 out of 13 transformants showed resistance against isolate EU-B05 at levels that were comparable to cv. 'Santana', a natural resistant cultivar. Two transformants, LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12*,* showed no sporulation and no symptoms. This resistance may be correlated with high expression of *HcrVf2* gene under long and rubisco promoters, respectively. The details are given in Figure 4. Similarly Malnoy et al. (2008) showed that *Vfa2* also gave partial resistance to mixed inoculum of *V. inaequalis*. They observed reduction in susceptibility to apple scab by 38%

for *Vfa2*. They explained partial resistance provided by *Vfa2* by the same possible reasons as for *Vfa1* partial resistance. Szankowski et al. (2009) studied expression of *HcrVf2* under the control of different lengths of native promoters namely 115 bp, 288 bp, 779 bp and the *nos* terminator. They found that eight out of nine transgenic lines were resistant against a mixture of *V. inaequalis* conidia. All the *HcrVf2* transformants with 288 bp and 779 bp promoters showed no symptoms after inoculating with *V. inaequalis*. Our results are in agreement with these results.

Along with the native promoters (short and long), the apple rubisco small subunit promoter was also used in testing the effect of expression of the *HcrVf1* and *HcrVf2* genes on resistance. *Ribulose-1, 5-bisphosphate carboxylase/oxygenase* (rubisco) is the primary enzyme of the carbon fixation process. It is the most abundant protein on earth and constitutes approximately 50-60% of total plant protein (Dean and Leech 1982). Expression is thought to be very high in green plant tissues and the rubisco small subunit promoter provided high level expression in heterologous systems in plants. In this study, P_{MdRbc}-regulated *HcrVf1* gene expression in the transgenic lines was on an average 350 times higher than *HcrVf1* expression in the reference resistant cultivar, 'Santana', driven by its native promoter in the natural situation. For P_{MdRbc}*HcrVf2*, gene expression was on an average 95 times higher in transgenic lines than *HcrVf2* expression level in cv. 'Santana'. Earlier experiments showed that the Soybean rubisco and Tomato rubisco promoters gave very high expression of the reporter gene *gus* in apple (Gittins et al. 2000). The chrysanthemum rubisco promoter used to express the *gus* gene in tobacco gave 7-8 fold higher expression than the constitutively expressed 35S promoter (Outchkourov et al. 2003). Also the apple rubisco promoter showed higher expression of the reporter gene *gus* in tobacco (Schaart et al. 2010b). The use of apple rubisco promoters for expressing *HcrVf2* in apple can be considered as transformation using only native DNA, which is being referred to as intragenics/intragenesis (Rommens 2004; Rommens et al. 2007). This type of genetic transformation will minimizes the content of foreign DNA, possibly making it more acceptable to consumers (Lusk and Rozan 2006), similarly to the cisgenic approach.

Correlation between sporulation and gene expression

The correlation between the expression data and the sporulation data was studied using Spearman's correlation co-efficient. In this study, an overall negative correlation was observed between the expression of *HcrVf2* and sporulation (Figure 4). Expression of wheat puroindoline-b (PinB) was studied for apple scab susceptibility in transgenic apple by Faize et al. (2004). They observed a strong negative correlation between PinB content and susceptibility towards the strain of race 6 i.e. increase in the PinB content decreased the

susceptibility of apple for scab disease. In our experiment some transformants, however, even though the expression was similar to cv. 'Santana', sporulation was similar to untransformed cv. 'Gala'. This can be hypothesized as may be mutation of the gene during the process of transformation resulting in non-functional gene.

Conclusion

In conclusion, two genes putatively conferring resistance to apple scab, i.e. *HcrVf1* and *HcrVf2*, were tested for expression and resistance with their native promoters at two sizes (SP and LP) and compared to their performance under control by the apple rubisco promoter. It was concluded that only *HcrVf2* represents a functional resistance gene for *V. inaequalis* isolate EU-B05. Long promoters gave higher expression than short promoters although the difference was not significant in case of *HcrVf2.* The apple rubisco promoter yielded the highest expression and combined with *HcrVf2* performed best for resistance. Negative correlation was demonstrated between expression levels of *HcrVf2* and sporulation levels of EU-B05 isolate, meaning the higher the expression, the more resistant.

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Chapter 5

Resistance spectrum of *HcrVf* genes to *Venturia inaequalis*

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Abstract

Apple scab caused by the fungus *Venturia inaequalis* is an epidemic world wide, especially in wet climates. This disease results in yield reduction and abnormal fruits and hence, economic loss. The crab apple *Malus floribunda* 821 which carries *Vf* scab resistance has been widely used in breeding programs to develop scab resistant cultivars. However, there have been reports on new isolates of *V. inaequalis* known to have overcome the *Vf* resistance.The *Vf* locus appears to carry four genes (*HcrVf1, HcrVf2, HcrVf3,* and *HcrVf4*). Two of these (*HcrVf1* and *HcrVf2*) have been designated to provide scab resistance. We developed apple transformants with *HcrVf1* or *HcrVf2* with a native short promoter (SP) of 312 bp and 288 bp respectively, a native long promoter (LP) of 1990 bp and 2000 bp respectively, and with the apple rubisco promoter (P_{MdRbc}). The SP and LP *HcrVf* genes are considered to be cisgenic and P_{MdRbc}*HcrVf* genes as intragenic. Based on the results of previous inoculation experiments with monoconidial scab isolate EU-B05, plant transformants were selected from all representative gene constructs to check the resistance spectrum of *HcrVf1* and *HcrVf2* to different, isolates of *V. inaequalis*. Individual transformants carrying either *HcrVf1* or *HcrVf2* genes were propagated and inoculated with four *Vf* avirulent isolates and two *Vf* virulent isolates separately. All the *HcrVf1* transformants showed sporulation irrespective of the isolates used or expression levels of *HcrVf1*. Generally, in *HcrVf2* transformants no sporulation was observed for the avirulent isolates, reduced sporulation of the virulent isolate EU-NL05 and high sporulation of the virulent isolate EU-D42 was observed. Even highly elevated levels of expression of *HcrVf2* as found in P_{MdRbc}*HcrVf2* transformants did not confer resistance against the *Vf* virulent isolate EU-D42. It was concluded that *HcrVf1* is not playing any role in conferring resistance to apple scab and that *HcrVf2* is the only functional scab resistance gene against the four avirulent isolates.

Introduction

Apple scab caused by the fungus *Venturia inaequalis* is a devastating disease on fruits and may result in reduction of yield and abnormal fruits leading to high economic loss. The pathogen is a heterothallic ascomycete with an annual cycle of sexual reproduction on infected apple leaves later followed by several asexual reproduction cycles during the growing season of apple (McHardy 1996; Xu et al. 2009). The ornamental apple *Malus floribunda* 821 which carries the *Vf* locus was widely used in breeding programs to develop scab resistant apple cultivars (Janick et al. 1996). The first known efforts on breeding for scab resistant cultivars date back to 1914 (Crandall 1926). The first cultivar carrying "*Vf*", named 'Prima', was released in 1970 (Calenge et al. 2004). However, further breeding was needed for improving fruit quality. And still, after nearly one century of breeding efforts, the quality is not yet as high as the commonly sold susceptible cultivars.

In 1988, on cv 'Prima', small scab lesions were observed at Ahrensburg, Germany and first time reported about a new race of *V. inaequalis* known to have overcome the resistance of *Vf* and they called it as race 6 (Parisi et al. 1993). Later, more *Vf* virulent isolates were found in several places in Europe (Parisi et al. 2004). Bus et al. (2009) described nine isolates based on gene-for-gene interaction and described new nomenclature of the scab resistance genes and complementary avirulence genes.

The pathogen's life cycle consists of one sexual cycle followed by many asexual cycles that lead to high genetic diversity within the population (Tenzer and Gessler 1999). This kind of reproductive system facilitates the fungus to adapt to specific resistance genes of the host by increasing the risk of avirulence losses and by subsequent rapid spreading of new virulences (Guérin and Cam 2004).

Calenge et al, (2004) studied resistance spectra of different QTL, using eight different isolates of *V. inaequalis.* Four out of eight isolates were virulent to *Vf.* They identified QTL for isolate specific (individual isolate) and broad spectrum resistance (giving resistance to seven out of eight isolates) on different linkage groups of the apple genome (Calenge et al. 2004). Durel et al. (2003) investigated inheritance of resistance in a 'Prima' x 'Fiesta' progeny by inoculating with two isolates namely EU-D42 and 302 of race 6 of *V. inaequalis*. They observed a QTL very close vicinity of the *Vf* resistance gene on linkage group 1.

The apple scab resistance locus *Vf* was sequenced and four paralogs were identified namely *HcrVf1, HcrVf2, HcrVf3,* and *HcrVf4* (Vinatzer et al. 2001). Xu and Korban (2002) reported similar results and predicted that only *HcrVf1* and *HcrVf2* are functional genes for scab resistance. The functionality of *HcrVf2* was studied by expressing under the regulation

of *CaMV35S* promoter and *nos* terminator and proved to show complete resistance against *V. inaequalis* (Belfanti et al. 2004). Malnoy et al. (2008) studied resistance against a mixture of strains (Race1 to Race 5) of *V. inaequalis*, when they transformed *Vfa1* (which is same as *HcrVf1*) and *Vfa2* (which is same as *HcrVf2*) with their native promoters and terminators, to susceptible cultivars 'Galaxy' and 'McIntosh' and showed partial resistance of *HcrVf1* and *HcrVf2* to the mixture. Szankowski et al. (2009) showed complete resistance by inserting only *HcrVf2* under the regulation of different lengths of native promoters and *nos* terminator into cv. 'Gala'. They used also a mixture of *V. inaequalis* strains for scab inoculation.

In this study, we are reporting on the use of six different isolates of *V. inaequalis* to study the spectrum of resistance provided by *HcrVf1* and *HcrVf2*. Plant transformants were developed by inserting *HcrVf1* and *HcrVf2* genes individually and were inoculated with four avirulent isolates and two virulent isolates, for *Vf*. To our knowledge this is first detailed study of two individual *Vf* resistance genes using different isolates of *V. inaequalis* to study their resistance spectrum.

Materials and methods

Plant transformation

Two resistance genes, *HcrVf1* and *HcrVf2*, were cloned with their native regulatory elements, *HcrVf1* short promoter (SP)-312 bp, *HcrVf1* long promoter (LP)-1990 bp, *HcrVf2* short promoter (SP)-288 bp, *HcrVf2* long promoter (LP)-2000 bp. They also contained the native terminator sequences of 480 bp and 437 bp for *HcrVf1* and *HcrVf2* respectively. All these genes with their own promoter are considered to be cisgenic (Schouten et al. 2006a). Rubisco is a highly expressed protein (Ellis 1979) and the apple rubisco promoter (P_{MdRbc}) (1600 bp) and apple rubisco terminator (T_{MdRbc}) (593 bp) (Schaart et al. 2010b) was also used to express *HcrVf1* and *HcrVf2* individually at very high levels. These genes are considered to be intragenic as described by Rommens et al. (2007). The susceptible cv. 'Gala' was used for plant transformation. Development of plant transformants carrying *HcrVf1* or *HcrVf2* and molecular analysis of these plants are given in detail in the Materials and Methods part of Chapter 3. Based on the results of previous inoculation experiments with the monoconidial isolate EU-B05, plant transformants were selected from all representative gene constructs to check the resistance spectrum of *HcrVf1* and *HcrVf2* to different scab isolates. Cvs 'Gala' and cv. 'Santana' were included as scab susceptible and *Vf* cultivar containing controls respectively. Cv. 'Santana' contains both *HcrVf1* and *HcrVf2* as a result of classical

breeding, and has *M. floribunda* 821 as an ancestor. The nature and number of plants used in the scab test are given in Table 1.

Micrografting

In vitro plant transformants SP*HcrVf1*-12, LP*HcrVf1*-7, P_{MdRbc}*HcrVf1*-9, SP*HcrVf2*-11, LP*HcrVf2*-16, P_{MdRbc}*HcrVf2*-3, LP*HcrVf2*-4, P_{MdRbc}*HcrVf2*-12 and the in vitro cvs. 'Santana' (resistant control) and 'Gala' (susceptible control) were used for micrografting onto apple rootstocks in the greenhouse according to the procedure as explained in the Materials and Methods part of Chapter 3. These micrografted shoots were used in scab resistance evaluation (Table 1). The temperature of 19°C during day and 16°C during night, and 16h daylight was maintained.

Inoculum

Six monoconidial isolates (genetically homogeneous) of *V. inaequalis* were used in the inoculation test. Isolates EU-B05, 1639, US-3, EU-D42, EU-NL05, and NZ188 were used to inoculate the plants. Isolates EU-B05, 1639, US-3 and NZ188 are avirulent to *Vf* and EU-D42 and EU-NL05 are virulent to *Vf* in cv 'Prima' (Calenge et al. 2004). The details of origin, isolate status, race status and new nomenclature (Bus et al. 2009) are given in Table 2.

Table 2. The origin, isolate and race status of *V. inaequalis* used to study the resistance spectrum of *HcrVf* genes to *V. inaequalis*

Origin	Isolate	Race status	New race status*
Belgium	EU-B05	1	(1, 3)
France	1639	2	(1, 2, 8, 9)
United States of America	US3	3	
Germany	EU-D42	6	(1, 6, 14)
Netherlands	EU-NL05	7	(1, 6, 7, 13)
New Zealand	NZ188	8	(1, 8)

* A new nomenclature of *V. inaequalis* has been proposed (Bus et al. 2009).

Each inoculum was prepared carefully to maintain the homogeneity of the isolate. To avoid contamination different isolate tubes were placed at fairly large distance on a table.

Table 1. Cultivars and plant transformants used to study the resistance spectrum of *HcrVf* genes to *V. inaequalis*

Genotypes/transformants	Inserted Gene copy number [3]	Expression of inserted gene[4] #	Resistance[4] against EU-B05	Number of plants tested per combination of event and isolate						
				EU-B05	1639	US-3	EU-D42	EU-NL05	NZ 188	Blank (Water)
'Gala'	-	0.00	S	4	3	4	4	3	4	3
'Santana'	-	1.00	R	4	3	4	4	4	4	-
SP*HcrVf1*-12	1	2.4	S	4	1	4	4	4	4	-
LP*HcrVf1*-7	1	13	S	4	3	4	4	4	4	-
P_{MdRbc}*HcrVf1*-9	2	421	S	4	-	4	4	4	4	-
SP*HcrVf2*-11	1	0.84	R	4	2	4	4	4	4	-
LP*HcrVf2*-16	2	1.1	S	4	3	4	4	4	4	-
P_{MdRbc}*HcrVf2*-3	1	81	R	4	3	4	4	4	4	2
LP*HcrVf2*-4	1	7.1	R	-	-	-	3	-	4	-
P_{MdRbc}*HcrVf2*-12	2	163	R	4	-	-	4	-	4	-

'Santana'- resistant control, 'Gala'- susceptible control, SP- short promoter, LP- long promoter, PMdRbc - apple rubisco promoter,
R- Resistant, S-Susceptible, # indicates expression of inserted genes measured in relation to expression in 'Santana' which is considered as 1.00,
3 the results obtained from Chapter 3, 4 the results obtained from Chapter 4

A few leaves (depending upon sporulation on those leaves in previous season) were put in a beaker with 40 ml of clean water and they were mixed gently and stirred every minute. The spore suspension was filtered using pre-wetted cheese cloth. The filtrate inoculum was used for inoculation. The concentration of inoculum was checked using a Haemocytometer. The concentration of inoculum was adjusted by adding water or leaves till a concentration of 5 x 10^5 conidia/ml was obtained. A few drops of these inocula were put in Petri dishes that were sealed with Parafilm. After 24 hours the drops of inocula were checked under the microscope to study the spore germination percentage. A spore germination rate of more than 90% was observed for each inoculum. Water was used as blank treatment. Due to non-availability of sufficient plant materials the blank (water) treatment was used only for cv 'Gala' and P_{MdRbc}*HcrVf2*-3.

Scab disease tests

Inoculation

Plants were randomized in blocks according to a Randomized Complete Block Design (RCBD) to get unbiased data during scoring. The top two young leaves were used for inoculation with the fungus. Inoculated plants were kept in a tunnel made by polyethylene plastic sheets and PVC framework for 48 hours at 20°C and 100% relative humidity, and then the plants were transferred outside the tunnel in the greenhouse with a temperature of 19°C during day and 16°C during night, relative humidity of 85% and daylight of 16hr.

Both quantitative (described by Durel et al. 2003) and qualitative (Chevalier et al. 1991; Szankowski et al. 2009) scales were used for scoring 21 days after inoculation. Quantitative scale scoring is a measure of sporulation of the fungus on the leaf surface (Chapter 4). Qualitative scale scoring is a measurement of symptoms on the leaves. The scale includes five different classes namely, class 0: no visible symptoms, class 1: pinpoint pits, class 2: yellow chlorotic flecks, class 3a: yellow chlorotic to necrotic flecks without sporulation, class 3b: necrotic flecks with sparse sporulation, and class 4: abundant sporulation.

Statistical analysis

As mentioned, the resistance test was performed according a Randomized Complete Block Design (RCBD). The genotypes and isolates were the experimental units. The number of replicates used in this experiment was four. In total 201 plants were used in this analysis. All the statistical analyses were performed using Genstat® 12 (Genstat® 2009). In some genotypes there were not equal number of plants per replication and there were not equal

number of plants for inoculation with some isolates (Table 1). Therefore the experimental setup became unbalanced, so linear mixed model (LMM) was fitted and used for analysis.

Results

Micrografting

All the micrografted shoots except of the lines LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12 developed with high efficiency. Due to small size of in vitro shoots that were used in case of LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12, fewer number of shoots were micrografted for these constructs. An overview of the number of micrografted shoots, the inserted gene copy number, expression of the *HcrVf1* and *HcrVf2* cisgenes and resistance to EU-B05 is given in Table 1.

Scab resistance evaluation

The complete sporulation pattern of different genotypes and transformants were presented in the Table 3. All the *HcrVf1* transformants showed heavy sporulation for all isolates. The sporulation levels of the *HcrVf1* transformant did not differ significantly to the sporulation level of the susceptible reference cv. 'Gala' (Susceptible control).

In *HcrVf2* transformants LP*HcrVf2*-16 showed similar sporulation level as cv. 'Gala' for all the tested fungal isolates. However, all other *HcrVf2* transformants showed a sporulation pattern similar to cv. 'Santana' (resistant control) for avirulent isolates, reduced sporulation for the virulent isolate EU-NL05 but showed heavy sporulation for the virulent isolate EU-D42. The promising plant transformant LP*HcrVf2*-4 was tested against isolates EU-D42 and NZ 188, and showed resistance against isolate NZ 188 (0.00) and sporulation of isolate EU-D42 (2.83). Another promising plant transformant P_{MdRbc}*HcrVf2*-12 was tested against isolates EU-B05, EU-D42 and NZ 188 and showed resistance against EU-B05 (0.00), and NZ 188 (0.00), and sporulation with EU-D42 (2.12). An overview of the sporulation on individual plant transformants of all the isolates of the fungus is presented in Table 3 and Figures 1 and 2.

The sporulation of different isolates as it was observed on leaves of cv. 'Santana', SP*HcrVf1*-12, P_{MdRbc}*HcrVf2*-12, and LP*HcrVf2*-4 has been shown in Figure 3.

In a graphic presentation of the data of Table 3 including means and standard errors two distinct groups of plants could be observed for all the isolates except EU-D42 (Figures 1 and 2). One group of plants showed sporulation levels similar to (not significantly different from) the sporulation of the susceptible control. The other group of plants showed similar sporulation as the *Vf*-cultivar 'Santana'. These two groups of plants were statistically significantly different from each other for all the used avirulent strains. These two groups can regarded as susceptible and resistant group respectively. In the case of cv. 'Gala', LP*HcrVf2*-16 and all *HcrVf1* transformants the sporulation was not significantly different from cv. 'Gala'. Among the cultivars and transformants there was no significant difference observed for the virulent isolate EU-D42.

The relation between expression of *HcrVf* genes and sporulation of different isolates was also studied (Table. 3). The expression level of *HcrVf* genes in transformants was measured in relation to the level of expression in cv. 'Santana' and is represented in the parenthesis of each genotype. In cv. 'Gala' due to the absence of scab resistance genes there was no expression and the sporulation was high irrespective of the isolates. In spite of the high expression of *HcrVf1* in transformants SP*HcrVf1*-12 (2.3), LP*HcrVf1*-7 (13) and P_{MdRbc}*HcrVf1*-9 (421), these plants showed sporulation level similar to cv. 'Gala' irrespective of the isolates tested. In cv 'Santana' (1), SP*HcrVf2*-11 (0.84), P_{MdRbc}*HcrVf2*-3 (81), P_{MdRbc}*HcrVf2*-12 (163), and LP*HcrVf2*-4 (7.1) expression of *HcrVf2* showed resistance against the avirulent isolates (EU-B05, 1639, US-3 and NZ 188) in the range of 0.1 to 0.7, reduced sporulation of virulent isolate EU-NL-5 (0.6 to 1.0) and complete sporulation of virulent isolate EU-D42 (3.3 to 3.6). Although in transformant LP*HcrVf2*-16 (1.1) *HcrVf2* gene expression was similar to the expression in cv. 'Santana', the plant showed sporulation level similar to cv. 'Gala' for all the isolates tested.

Table 3. Means and their standard deviations of sporulation of different isolates on genotypes and transgenic lines

Race / Genotypes	Expression of inserted gene[4] #	EU-B05	1639	US-3	EU-D42	EU-NL05	NZ 188
'Gala'	0.00	2.87±1.97	4.16±2.02	4.00±0.81	3.62±1.75	3.16±1.04	5.00±0.40
'Santana'	1.00	0.37±0.25	0.16±0.28	0.25±0.50	3.37±1.79	1.00±0.00	0.37±0.47
SP*HcrVf1*-12	2.4	3.50±1.77	5.0	5.00±0.57	2.87±1.65	3.50±0.40	4.87±0.62
LP*HcrVf1*-7	13	2.37±0.85	4.16±0.76	4.50±0.70	2.87±1.88	3.87±0.85	5.25±0.28
P_{MdRbc}*HcrVf1*-9	421	2.62±0.85	NA	4.50±1.22	2.62±0.62	3.12±0.94	4.00±1.08
SP*HcrVf2*-11	0.84	0.25±0.28	1.00±1.41	0.62±.94	3.62±0.85	0.62±0.25	0.75±0.28
LP*HcrVf2*-16	1.13	4.00±1.22	4.30±0.76	4.37±1.31	3.62±1.03	2.37±0.47	3.62±1.65
P_{MdRbc}*HcrVf2*-3	81	0.00±0.00	1.50±0.91	0.62±0.75	3.37±1.97	0.62±0.47	0.41±0.58
P_{MdRbc}*HcrVf2*-12	163	0.00±0.00	NA	NA	2.12±1.03	NA	0.00±0.00
LP*HcrVf2*-4	7.1	NA	NA	NA	2.83±1.60	NA	0.00±0.00

Races are represented in horizontal direction, Genotypes are represented vertically, SP-Short Promoter, LP- Long Promoter, P_{MdRbc}- Apple Rubisco promoter, 'Santana' - resistant cultivar, 'Gala' - susceptible cultivar, NA- no plants available, # indicates expression of inserted genes measured in relation to Santana which is considered as 1.00, [3] the results obtained from Chapter 3, Means represent average of two leaves of four plants (eight values), ± represents the standard deviation of the mean. Sporulations were scored based on scale as described by Durel et al. (2003) which is class 0, 0% of sporulation; class 1, 1–2% sporulation; class 2, 2–5% sporulation; class 3, 5–10% sporulation; class 4, 10–25% sporulation; class 5, 25–50% sporulation; class 6, 50–75% sporulation; class 7, 75–100% sporulation.

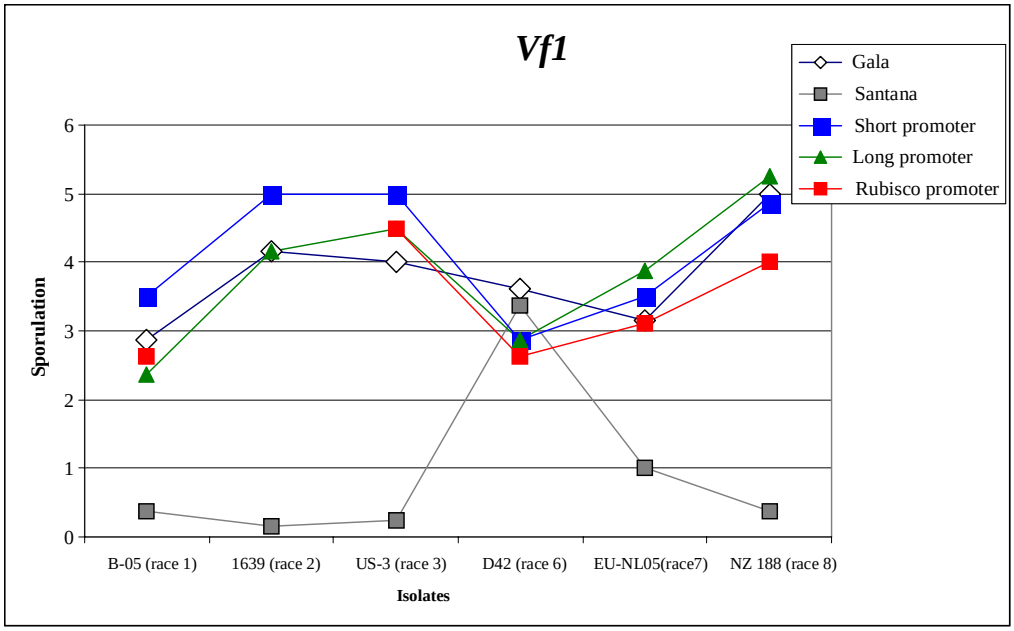

Figure 1. Sporulation of different isolates of *V. inaequalis* on genotypes containing the *HcrVf1*. Sporulation is average of two leaves of four plants (eight values). B-05- EU-B05 and D42-EU-D42.

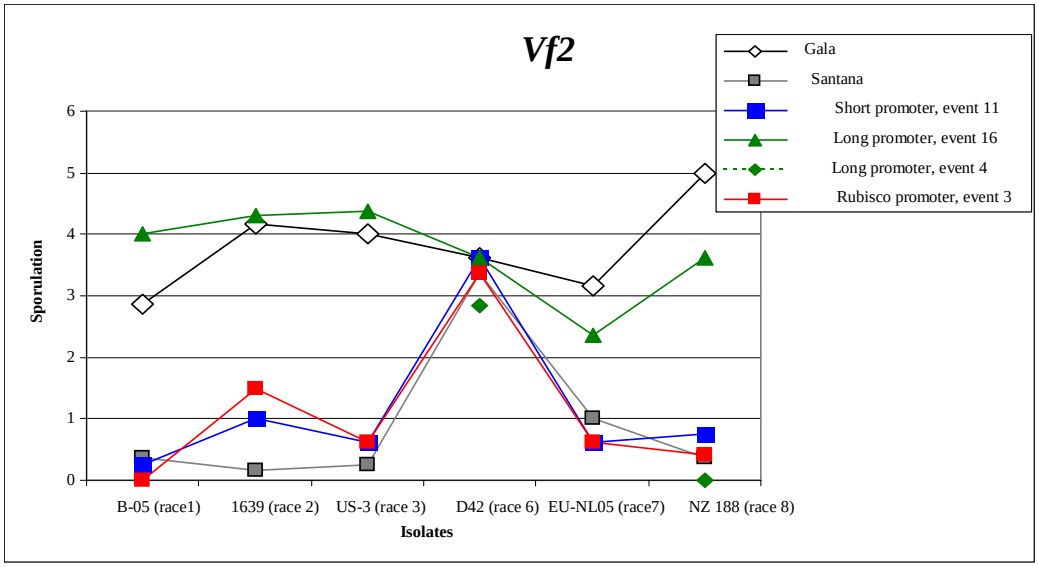

Figure 2. Sporulation of different isolates of *V. inaequalis* on genotypes containing the *HcrVf2*. Sporulation is average of two leaves of four plants (eight values).

Figure 3. Performance of Santana, SP*HcrVf1*-12, P$_{MdRbc}$*HcrVf2*-12, LP*HcrVf2*-4 in symptom development against different isolates of *V. inaequalis*

Discussion

In our previous experiment we have demonstrated that *HcrVf2* is the only functional gene against isolate EU-B05 and we found that *HcrVf1* does not play any role in conferring resistance against isolate EU-B05 (Chapter 4).

Six isolates, of which four are avirulent strains and two are virulent strains of *V. inaequalis* for cv. 'Santana' and other *Vf* based resistant varieties, were used to test the resistance spectrum of the separate *HcrVf1* and *HcrVf2* genes. Since in the natural situation *HcrVf1* and *HcrVf2* inherit as one locus, it has not been possible earlier to study the role of the individual *HcrVf1* and *HcrVf2* genes in conferring resistance to different isolates of apple scab when classically bred *Vf* cultivars were used. In order to study the spectrum of *HcrVf1* and *HcrVf2* resistance, we have developed apple transformants with *HcrVf1* under the influence of a short (SP) and long promoter (LP) apple rubisco promoter (P_{MdRbc}) and *HcrVf2* under the influence of SP, LP, and P_{MdRbc} separately. As far as we know this work, to study the resistance spectrum of *HcrVf1* and *HcrVf2* with different isolates is the first of its kind.

Scab resistance evaluation

Since cv. 'Gala' (susceptible control) does not harbor any known effective resistance gene to *V. inaequalis* it showed high levels of sporulation by all used isolates (Belfanti et al 2004; Szankowski et al. 2009). All *HcrVf1* transformants also showed similarly high sporulation with all isolates, demonstrating complete susceptibility (Figure 1). There was clear indication that all the *HcrVf1* transformants behaved similar as the untransformed cv. 'Gala' (Figure 1). This was in accordance with the earlier observation for the same transformants when inoculated with isolate EU-B05 (Chapter 4). This result with *HcrVf1* is however in contrary to the result obtained by Malnoy et al. (2008). They inserted *Vfa1* (which is the same as *HcrVf1*) and *Vfa2* (which is the same as *HcrVf2*) under their native promoters of 2 kb length and their native terminator, 1 kb of length, into cvs. 'Galaxy' and 'McIntosh'. They used a mixed inoculum representing races 1 to 5 and they observed partial resistance in both cases of *HcrVf1* and *HcrVf2*. They explained that this partial resistance may be either due to a high concentration (2.7×10^7 conidia/ml) of fungal inoculum or mutation of *Vfa1* and *Vfa2* genes during the different steps of transformation process or to the physical separation of *HcrVf1* and *HcrVf2*, which in the natural situation exist together.

Cv. 'Santana' which harbors both *HcrVf1* and *HcrVf2* in the natural situation, SP*HcrVf2*-11, and P_{MdRbc}*HcrVf2*-3 showed no sporulation with fungal isolates EU-B05,

1639, US-3, and NZ 188, while sporulation with EU-D42 and EU-NL05 did occur, proving susceptibility to these virulent isolates confirming results from Parisi et al. (1993). It is clear that *HcrVf2* transformants are clearly behaving like cv 'Santana' (Figure 2) indicating that the resistance coming from *Vf* cluster is from *HcrVf2*. However, the level of sporulation of the virulent strain EU-NL05 was rather low (Table 3).

From the *HcrVf2* transformants, LP*HcrVf2*-16 was completely susceptible to all isolates confirming the results of the earlier spring experiment (Chapter 4). Presence of the *HcrVf2* gene was demonstrated in this line and expression was at a similar level to the expression in cv. 'Santana'. There can be several explanations for its susceptibility. Firstly it could be hypothesized that mutation of the gene during transformation led to susceptible phenotype. Secondly could be due to no induction of the *HcrVf2* gene after pathogen identification. In our preliminary experiment we observed there was no difference in transcript level before and after inoculation (data not shown). It may be this failure in induction of the gene after perceiving the pathogen has resulted in sporulation. This possible lack of induction may have been caused by lack of recognition of the pathogen, or a disruption in the signal transduction in the plant, or a change in the epigenetic status of the promoter of *HcrVf2* such as methylation, preventing upregulation.

The two most promising apple transformants which gave very high resistance in the previous scab experiment (Chapter 4) and very high gene expression (Chapter 3), are LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12 and they showed only sporulation with isolate EU-D42. LP*HcrVf2*-4 showed no sporulation with isolate NZ 188 and P_{MdRbc}*HcrVf2*-12 showed no sporulation for isolates EU-B05 and NZ 188. This indicates that the isolate EU-D42 lacks avirulence (*AVR*) proteins that are recognized by the *HcrVf2* gene. The reason for non recognition of *AVR* genes may be either due to mutation or silencing of *Vf-AVR* gene in the isolate EU-D42. Other explanation could be the isolate has these *AVR* genes, but is able to block the defense reaction. However, the *Vf* virulent isolate EU-D42 was confirmed to be virulent. Even 7- and 163-fold increase in the gene expression compared to cv. 'Santana' as were found in LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12 respectively (Chapter 3), could not help in providing resistance against this virulent isolate.

Among all lines and cultivars checked two clear groups could be distinguished except for isolate EU-D42 (Figures 1 and 2). All *HcrVf1* transformants, LP*HcrVf2*-16 and cv. 'Gala' were grouped together in a distinct susceptible group being significantly different from the group of all the other *HcrVf2* transformants and cv. 'Santana' that can be designated as resistant. It was seen from the Table 3 and Figures 1 and 2 that *HcrVf1* transformants clearly followed the pattern of untransformed cv 'Gala' and *HcrVf2* transformants clearly

followed the pattern of cv. 'Santana'. This is a strong indication that the resistance coming from *Vf* cluster is entirely and solely due to *HcrVf2*.

In cv. 'Santana', SP*HcrVf2*-11, and P_{MdRbc}*HcrVf2*-3 sporulation with EU-NL05 was observed, but at a significantly reduced level compared to cv. 'Gala'. As for the avirulent isolates the two earlier identified groups could also be distinguished here (Figure 2). It can be hypothesized that this isolate is partially virulent to *Vf*, but not fully as seen in isolate EU-D42. The expression of *HcrVf2* in cv. 'Santana' and in SP*HcrVf2*-11 (similar expression as cv. 'Santana') and in P_{MdRbc}*HcrVf2*-3 [much higher expression than cv. 'Santana' (Chapter 3)] was sufficient to give the observed improved response to EU-NL05.

Relation between expression and sporulation

The relationship between the expression of *HcrVf* genes and sporulation of different isolates was also studied (Table 3). Even though upto to 420 times higher expression of *HcrVf1* in transformants than expression in cv. 'Santana' could not able to provide resistance against any of the isolates. This result was in accordance with the result obtained in the previous inoculation experiment (Chapter 4) indicating that the *HcrVf1* is not a functional scab resistance gene against any of the isolates tested.

In cv. 'Santana', SP*HcrVf2*-11, P_{MdRbc}*HcrVf2*-3, P_{MdRbc}*HcrVf2*-12, and LP*HcrVf2*-4 gene expression provided resistance against avirulent isolates. But very high expression of the gene compared to cv. 'Santana' in P_{MdRbc}*HcrVf2*-3, P_{MdRbc}*HcrVf2*-12, LP*HcrVf2*-4 could not able to provide resistance against virulent isolate EU-D42. This indicates that not only the level of expression is important but other functional scab resistance genes are needed.

Future work

The future work involves removing of the selection markers that have been used for the selection of the SP*HcrVf2*, LP*HcrVf2* and P_{MdRbc}*HcrVf2* transformants and thus making them marker-free resulting in the generation of cisgenic and intragenic plants. The earlier intended stacking of *HcrVf1* and *HcrVf2* is not required anymore as *HcrVf1* proved to be not involved in conferring resistance against any of the isolates. The transformants SP*HcrVf2*-11 and P_{MdRbc}*HcrVf2*-11 are being made selection marker free.

Conclusion

In conclusion, two genes *HcrVf1* and *HcrVf2* under the regulation of their native promoter (SP and LP) and the apple rubisco promoter were studied for resistance against six

isolates of *V. inaequalis*. It was proven that the resistance provided by the *Vf* cluster is from *HcrVf2* across different isolates of *V. inaequalis*. *HcrVf1* does not confer resistance against any used isolate. Increasing the *HcrVf2* gene expression to high levels will not help in conferring resistance against virulent isolates.

So, the *HcrVf2* gene is the best choice for development of cisgenic and intragenic plants to get good resistance against avirulent isolates. In order to get even better and more durable resistance we need more resistance genes giving a broad spectrum resistance against avirulent and virulent isolates. For introduction of such combinations of multiple genes classical breeding, cisgenesis or intragenesis approach can be employed.

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Chapter 6

Identification and mapping of the novel apple scab resistance gene *Vd3*

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Abstract

Apple scab, caused by the fungal pathogen *Venturia inaequalis*, is one of the most devastating diseases for the apple growing in temperate zones with humid springs and summers. Breeding programs around the world have been able to identify several sources of resistance, the *Vf* from *Malus floribunda* 821 being the most frequently used. The appearance of two new races of *V. inaequalis* (races 6 and 7) in several European countries that are able to overcome the resistance of the *Vf* gene put in evidence the necessity of the combination of different resistance genes in the same genotype (pyramiding). Here we report the identification and mapping of a new apple scab resistance gene (*Vd3*) from the resistant selection '1980-015-25' of the apple breeding program at Plant Research International, The Netherlands. This selection contains also the *Vf* gene and the novel *V25* gene for apple scab resistance. We mapped *Vd3* on linkage group 1, 1 cM to the south of *Vf* in repulsion phase to it. Based on pedigree analysis and resistance tests, it could be deduced that '1980-015-25' had inherited *Vd3* from the founder 'D3'. This gene provides resistance to the highly virulent EU-NL-24 strain of the race 7 of *V. inaequalis* capable of overcoming the resistance from *Vf* and *Vg*.

Key words: *Venturia inaequalis, Malus x domestica, SSR, DArT markers*

Introduction

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G.Wint., is one of the most devastating diseases for apple (*Malus x domestica* Borkh) in temperate zones with humid growing seasons. Most of the commercial apple cultivars are susceptible to the disease, and growers have to spray 20-30 times with fungicides in a season. The use of resistant cultivars could reduce the cost to the growers and may also contribute to a cleaner environment and to a reduction of fungicide residuals on apples for consumers.

The most widely used apple scab resistance gene is *Vf* from *Malus floribunda* 821. However, in the mean time, *V. inaequalis* strains have been detected that are able to overcome the *Vf* resistance (Parisi et al. 1993). Especially in North-western Europe these strains are present and have spread around (Parisi et al. 2006). As a result, several orchards consisting of *Vf*-cultivars have to be sprayed like orchards with susceptible cultivars (Trapman 2006). For durable resistance several resistance genes should be accumulated (pyramiding). Fortunately, new loci which include both major genes and QTLs that confer resistance to a broad spectrum of *V. inaequalis* strains have been discovered in *Malus* (Calenge et al. 2004; Schmidt and Van de Weg 2005; Gessler et al. 2006; Gardiner et al. 2006). Until now 11 major apple scab resistance genes have been mapped (Table 1). Some of them are considered as ephemeral resistance genes, acting only against a very narrow spectrum of races of *V. inaequalis*. Molecular markers linked to these genes are available (Gessler et al. 2006), and in this context marker assisted selection (MAS) could be a useful tool in order to accelerate the breeding programs for example selecting the parents for the future crosses.

In this work we report the identification and mapping of a new qualitative apple scab resistance gene named *Vd3* from the selection '1980-015-25' of the apple breeding program of Plant Research International in Wageningen, The Netherlands. This gene provides resistance to the highly virulent EU-NL-24 strain of the race 7 of *V. inaequalis* which has overcome the resistance from *Vf* and *Vg*.

Material and methods

Plant Material and DNA extraction

For the mapping of *Vd3* we used the population 2000-012C (Table 2). This population is a part of the population 2000-012 comprising 894 individuals and derived from the cross between the scab resistant selection '1980-015-025' and the susceptible selection '1973-001-041'. In the resistance tests the cultivars 'Elstar', 'Priscilla', 'Gala', 'Golden

Delicious', the selections 'D3', '1980-015-025' and '1973-001-041' and some individuals of the population 2000-012 carrying only *Vd3* were used. For pedigree analysis, the apple cultivars 'Elstar' and 'Priscilla' and the selections 'D3', '1972-010-33' and '1980-015-025' were tested with the simple sequence repeat (SSR) CH-*Vf1*.

DNA extraction was performed from unfolded leaves of apple as described for the Diversity Arrays Technology (DArT) technique (Jaccoud et al. 2001) in http://www.diversityarrays.com.

Evaluation of scab resistance

Scab resistance was evaluated after tunnel tests with mist evaporation, where the four youngest leaves of six replicates of the progeny 2000-012 containing *Vd3* only, and reference cultivars ('Elstar', 'D3', 'Priscilla', 'Gala', 'Golden Delicious', '1980-015-025' and '1973-001-041') (Table 3) were inoculated with a monoconidial

Gene	Source of Resistance	Linkage group	Reference[1]
Va	Antonovka PI172623	1	Hemmat et al. (2003)
Vb	Hansen's baccata #2	12	Erdin et al. (2006)
Vbj	*Malus baccata jackii*	2	Gygax et al. (2004)
Vd	Durello di Forli	10	Tartarini et al. (2004)
Vf	*Malus floribunda* 821	1	Maliepaard et al. (1998)
Vg	Golden Delicious	12	Durel et al. (2000)
Vh2	*Malus pumila* R12740-7A	2	Bus et al (2005a)

Table 1. Major apple scab resistance genes that have been positioned on the genetic linkage map of *Malus*.

Vh4	*Malus pumila* R12740-7A	2	Bus et al (2005a)
Vh8	*Malus sieversii*	2	Bus et al (2005b)
Vm	*Malus pumila* R12740-7A	17	Patocchi et al. (2005)
Vr2	GMAL 2473	2	Patocchi et al. (2004)

[1]First report of the map position

Table 2. The segregating population 2000-012C used in this study

Resistance donor	Cross date	Evaluation date	N	Resistance/ Susceptible	$\chi 2$ (p value) mono ratio[1]	$\chi 2$ (p value) di ratio[2]
1980-015-025	2000	2006	92	41/51	1.08 (0.30)	18.71 (0.00)

[1] Goodness of fit for monogenic inheritance.

[2] Goodness of fit for digenic inheritance.

suspension of the 9 different races of *V. inaequalis* (10^5 conidia/ml) used in the disease tests (Table 3). These isolates belong to the European collection of *V. inaequalis* from the "Durable Apple Resistance in Europe" project (Lespinasse et al. 2000). Plants were incubated during 48 h at 20°C and 100% relative humidity, and then transferred to a greenhouse with a relative humidity of 85-90%. Disease symptoms were assessed macroscopically after 14-17 days indicating the presence (susceptible plant) or absence (resistant plant) of sporulation. The population 2000-012C was inoculated a few weeks after emergence of the young seedlings. For mapping purpose the strain EU-NL-24 was used. *V25* confers resistance to this strain. This hampered mapping of the other resistance gene, i.e. *Vd3*. Therefore the 143 plants that contained *V25* were discarded based on flanking markers of *V25*. The remaining 92 plants were used for mapping *Vd3*. EU-NL-24 is capable of overcoming the *Vf* gene (Parisi et al 2004) and combines the virulences of races 6 and 7 (Calenge et al. 2004).

Disease symptoms were assessed macroscopically after 14-17 days and rated in eight classes indicating the amount of sporulation as follows: class 0: 0% of sporulation; class 1: 1-2% sporulation; class 2: 2-5% sporulation; class 3: 5-10% sporulation; class 4: 10-25% sporulation; class 5: 25-50% sporulation; class 6: 50-75% sporulation; class 7: 75-100% sporulation. This scale is similar to that reported by Durel et al. (2003) which was adapted from Croxall et al. (1952). The two youngest inoculated leaves were scored for sporulation.

DArT markers

DArT markers were produced by Diversity Arrays Technology (Yarralumla, Australia) as described in Wenzl et al. (2004) and Wittenberg et al. (2005).

Table 3. Strains of *V. inaequalis* used in the disease tests, and their sporulation on cultivars and selections containing *Vf, V25* or *Vd3*. (R) indicates the absence or very low levels of sporulation and (S) indicates abundant sporulation. GD: 'Golden Delicious'; 041: '1973-001-041'; 025: '1980-015-025'.

Strain (race)	Without *V25*, *Vd3*, *Vf*				*Vf*	*V25* + *Vd3*+ *Vf*	*V25* + *Vd3*	*Vd3*
	Elstar	Gala	GD	041	Priscilla	025	D3	2000-012
EU-B05 (1)	S	S	S	S	R	R	R	S
EU-NL19 (1)	S	S	S	S	R	R	R	S
1639 (2)	S	S	S	S	R	R	R	S
US2 (3)	S	S	S	S	R	R	R	S
1638 (4)	S	S	S	S	R	R	R	S
EU-D42 (6)	S	S	S	S	S	R	R	S
EU-NL05 (7)	S	S	R[1]	S	S	R	R	S
1066 (7)	S	S	R[1]	S	S	R	R	S
EU-NL24 (7)	S	S	S	S	S	R	R	R

[1] The resistance against these two strains is due to the *Vg* gene (Bénaouf and Parisi 2000; Parisi et al. 2004)

SSR markers

All of the SSRs in linkage group (LG) 1 available at the High-quality Disease Resistant Apples for a Sustainable Agriculture (HiDRAS) database (http://www.hidras.unimi.it/) were selected. In total nine SSRs were screened in the population 2000-012C (Table 4). SSR amplifications were performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 µl, containing 75 mM Tris–HCl, pH 8.8; 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.5 µM of fluorescent dye-labelled forward primer (Hex or 6-Fam, Biolegio, Nijmegen, The Netherlands); 0.5 µM of reverse primer; 20 ng of genomic DNA; and 1 U of SuperTaq DNA polymerase (HT Biotechnology Ltd, Cambridge, UK) using the following temperature profile: 94°C for 2 min 30 s, then 34 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, finishing with 72°C for 5 minutes. Samples were analyzed on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) and scored with GENOTYPER version 3.6 (Applied Biosystems).

Table 4. SSRs tested in '1980-015-025' parent, their position in genetic maps of Discovery (Silfverberg-Dilworth et al. 2006) and '1980-015-025', and the $\chi 2$ statistical analysis of the segregation for monogenic inheritance.

SSR marker	Discovery Map (cM)	1980-015-025 Map (cM)	$\chi 2$	SSR origin
CH03g12	4.2	0.0	0.19	Liebhard et al (2002)
Hi21g05	7.6	-	1.35	Silfverberg-Dilworth et al. (2006)
Hi02c07[1]	23.1[4]	-	-	Silfverberg-Dilworth et al. (2006)
Hi12c02	42.0	31.7	1.90	Silfverberg-Dilworth et al. (2006)
NZ03c1[2]	53.5[4]	-	-	Guilford et al. (1997)
KA4b	42.6	34.4	0.55	Silfverberg-Dilworth et al. (2006)
CH-*Vf1*	55.9	37.4	0.04	Vinatzer et al. (2004)
Hi07d08[3]	67.8[4]	-	2.84	Silfverberg-Dilworth et al. (2006)
CH05g08	77.4	58.9	4.54	Liebhard et al (2002)

[1]Monomorphic marker in population 2000-012C
[2]Unclear pattern
[3]Mapped in the susceptible parent (data not shown)
[4]Not Mapped in 'Discovery'. Position in the 'Fiesta' map

Testing of specific markers linked to other scab resistance genes in LG1

RAPD marker P-136

The *Va*-linked RAPD marker P-136 reported by Hemmat et al. (2003) was also analyzed in the population 2000-012C to test its association with *Vd3*. RAPD amplification was performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) as described in Hemmat et al. (2003). PCR products were separated on 2% agarose gels (Hispanagar, Burgos, Spain).

Vf2ARD marker

The *Vf2ARD* marker developed by Boudichevskaia et al. (2008) was tested in the population 2000-012C. The PCR was performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) as described in Boudichevskaia et al. (2008). PCR products were separated on 1% agarose gels (Hispanagar).

Linkage analysis

The linkage analysis was carried out using JoinMap 4.0 software (Van Ooijen 2006) with the Kosambi mapping function (Kosambi 1944) used to convert recombination units into genetic distances. Linkage groups were established using as threshold a minimum logarithm of odds (LOD) of 6.0 and a recombination frequency lower than 0.4. The *Vd3* gene was mapped as a dominant gene based on the phenotype data (1 for Resistance and 0 for Susceptibility). The genetic linkage map was constructed for the resistant parent following the "two way pseudo test-cross" model of analysis (Grattapaglia and Sederoff 1994) and setting a "cross-pollinator" data type.

Results

Evaluation of scab resistance

The population 2000-012C, segregating for *Vf, V25* and *Vd3*, was used to evaluate the resistance against the monocodial strain of *V. inaequalis* EU-NL-24, capable of overcoming the resistance conferred by the *Vf* gene. The other strains used in the disease test overcame the resistance conferred by the *Vd3* gene (Table 3). The resistance donor was the selection '1980-015-025' that contains the *Vf, V25* and *Vd3* genes. This selection was heterozygous for the *Vd3* trait and the susceptible parent was homozygous recessive. From 14 to 17 days after inoculation, plants were scored for scab resistance and classified into eight classes based on symptoms (see "Material and Methods" section). As the monoconidial strain is virulent to the *Vf* resistance, two effective major resistance genes were left, i.e. *V25* and *Vd3*. *V25* and *Vd3* inherited independently. For the purpose of mapping *Vd3* precisely, 143 progeny plants carrying the *V25* gene were discarded using markers that flanked this gene tightly (unpublished data). The sporulation of the remaining seedlings (N=92) is depicted in the histogram in Figure 1. The resistance reaction observed in the plants carrying *Vd3* was a hypersensitive pit type and chlorotic reactions.

Based on this histogram, those non-sporulating plants were designated as resistant while those sporulating plants were designated as susceptible. On the basis of this scoring 41 out of the 92 F_1 individuals were considered resistant and 51 susceptible (Table 2). Segregation of resistance and susceptibility in this cross fitted the ratio of 1:1, based on the Chi-square test ($\chi 2 = 1.08$) using a significance level of p-value of 0.05, indicating a monogenic inheritance. The hypothesis of two independent major dominant genes involved in the resistance was also tested, but it was rejected due to the high values of the Chi-square test (18.71) (Table 2).

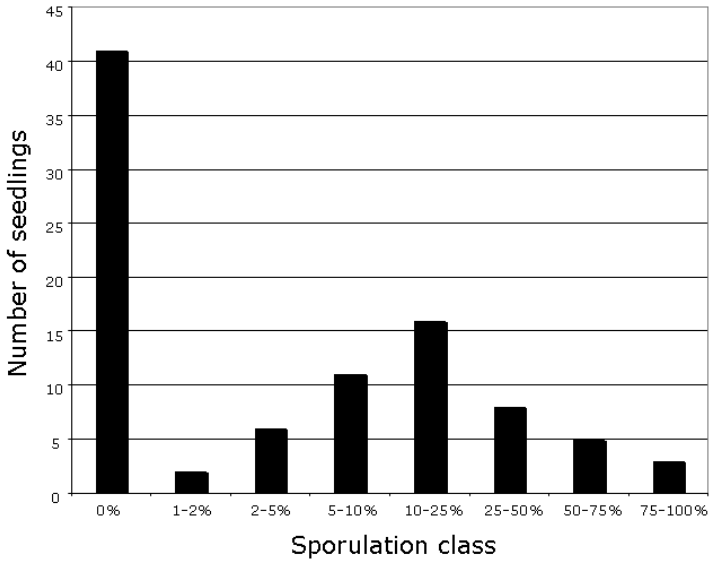

Figure 1 Histogram showing the number of young seedlings of population 2000-012C belonging to each one of the sporulation classes. The sporulation refers to percentage sporulating area in the upper two inoculated leaves

Construction of the LG1 of '1980-015-025'

This new gene could be mapped on LG1, closely to *Vf*, but on the homologous chromosome, so in repulsion to the CH-*Vf* marker (Vinatzer et al. 2004). For further mapping of *Vd3* SSRs in LG1 were tested. Seven out of nine SSRs screened were polymorphic in the mapping population and five of them were incorporated to the '1980-015-025' map (Table 4). In the case of the DArT markers, out of 234 markers from the resistant parent and absent in the susceptible parent, five were mapped on LG1. Other markers linked to scab resistance genes in LG1 were tested in population 2000-012C. With the first one, the RAPD marker P-136 linked to the *Va* gene according to Hemmat et al. (2003) should give the band of 700bp, but this band was not present in the population 2000-012C (data not shown). The second marker was the marker *Vf2ARD* developed by Boudichevskaia et al. (2008) based on the sequence of *HcrVf2* (Vinatzer et al. 2001) in the apple accessions 'Antonovka', 'Realka' and 'Discovery'. It was mapped in a similar location to the north of *Vf,* as reported by these authors (Figure 2), but the marker was also present in the susceptible parent of our population (data not shown).

At first LG1 was created only with SSR and DArT markers (Figure 2a). This map covers a distance of 58.9 cM comprising 11 loci. The SSRs mapped maintain the colinearity

with the 'Discovery' map of Silfverberg-Dilworth et al. (2006) and with the 'Florina' map reported by Broggini et al. (2009). Finally, *Vd3* was mapped on LG1 as a dominant heterozygous gene, using the resistance data of the segregating population (Figure 1). In a first location, this gene mapped between the SSR CH-*Vf1* and the DArT 67005F17 at a distance of 2.9 cM from the *Vf* locus (Figure 2b). After careful observation of the resistant/susceptible phenotype and genotype of the markers around *Vd3* it was observed that three progeny plants (one belonging to class 1 and two to class 2) showed genotype-phenotype incongruence (GPI) (Gygax et al. 2004). These plants, classified as susceptible, showed either marker alleles in coupling with the resistance allele. In this case by removing the resistance data of these plants *Vd3* was mapped again between the markers CH-*Vf1* and 67005F17, but the distance to the *Vf* locus was reduced to a 1.0 cM (Figure 2c). Exclusion of the GPI plants did not change the order of the markers, and the length of the LG 1 was only reduced 0.2 cM.

Pedigree analysis of '1980-015-025' and resistance source of *Vd3*

Figure 3 shows the parents and grandparents of the selection '1980-015-025'. The male parent 'Priscilla' was used as donor of the *Vf*-resistance. This could be confirmed with the 159 bp allele from CH-*Vf1* linked to *HcrVf1* (Vinatzer et al. 2004). This band was only present in the *Vf* cultivar 'Priscilla' and in '1980-015-025'.

As 'D3' appeared to be completely resistant in the field, this founder was used by the breeders of Plant Research International as donor of additional resistance. They crossed it with the susceptible cultivar 'Elstar'. 'Elstar' and 'Priscilla' are susceptible to the strain EU-NL-24 (Table 3), and thus lack the *Vd3* gene. However, 'D3' is resistant to EU-NL-24 (Table 3). This suggests that *Vd3* comes from 'D3'. This could be confirmed with the alleles of the CH-*Vf1* SSR, as shown in Fig 3: *Vf* co-segregates with the 159bp band of the SSR, whereas *Vd3* co-segretates with the 129bp band. The latter band originates from the source of resistance 'D3'.

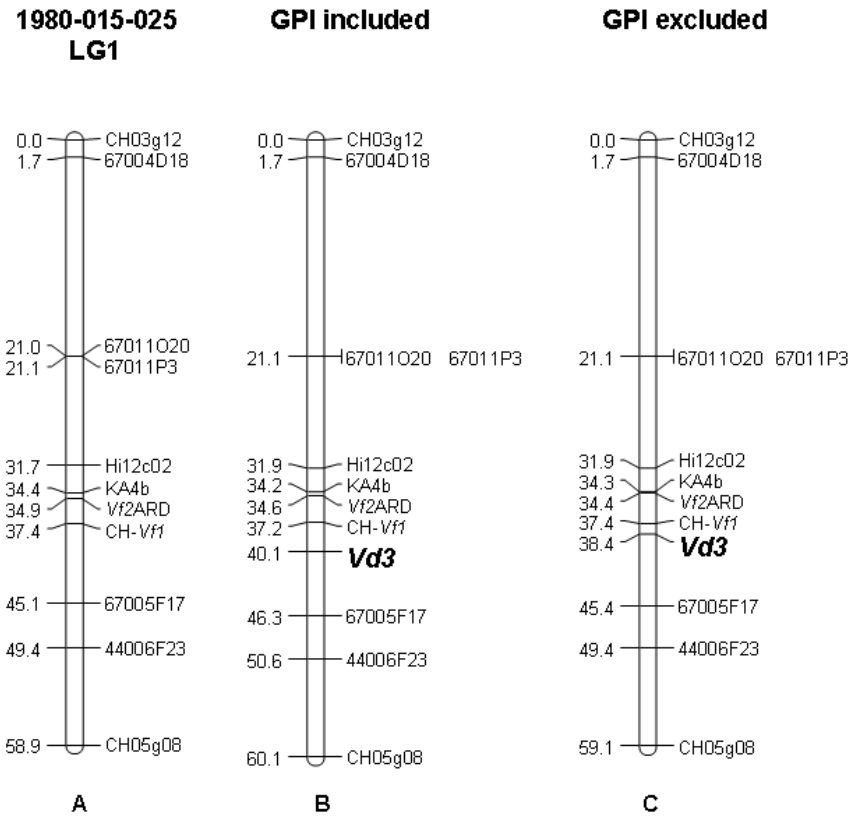

Figure 2 Genetic maps of *Vd3*. a) LG1 of '1980-015-025' showing the estimated position of *Vf* and *Va* genes according to Gessler et al. (2006). b) Map position of *Vd3* in LG1. c) Map position of *Vd3* after removing the phenotype information of the three genotype-phenotype incongruent (GPI) plants

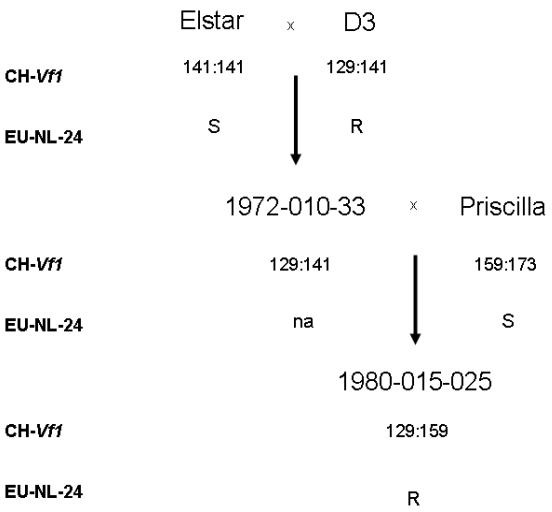

Figure 3 SSR and resistance test of the pedigree of '1980-015-025'. For the SSR CH-*Vf1* the numbers indicate the different alleles of the marker in bp and for the strain EU-NL-24. 'S' indicates the presence of sporulation and 'R' indicates the absence of sporulation. 'na' indicates non assessed

Discussion

Resistance screening

The correct placement of the threshold between resistance and susceptibility is very important to determine the resistance inheritance model and the position of the resistance gene in the linkage map. The selection of the threshold used in this work was due to the degree of sporulation observed from class 1 to class 7 and the absence of *V. inaequalis* spores in class 0 (Figure 1). Comparison with the genetic markers indicates that this threshold gives good results, apart from three seedlings with 1 – 5 % of sporulation. This indicates that *Vd3* usually provided complete resistance, but 3 % of the seedlings showed a low level of sporulation in spite of the presence of *Vd3*. We did not observe any healthy plants lacking *Vd3*. This implies that we had no plants that escaped from inoculation.

Linkage map

An accurate phenotyping of the resistance/susceptible data in the progenies is essential for the correct placement of the gene of interest in the linkage map. Two positions are proposed for the *Vd3* gene according to the inclusion or exclusion of GPI plants of the analysis (Figures 2b and 2c). In both situations the gene is mapped between the same

markers and the order of the markers in the map is not altered, but in the case of the exclusion of the GPI plants the distance between *Vd3* and the *Vf* locus was reduced from 2.9 to 1 cM. As was reported by other authors (Patocchi et al. 1999a; Gygax et al. 2004; Erdin et al. 2006), we found difficulties determining the correct position of *Vd3*, as the phenotypic data were not as precise as the molecular data. During the analysis of the population 2000-012C we classified 3 plants (3 %) as susceptible but the flanking marker alleles were in coupling with the resistance. One susceptible plant without *Vf* showed from 2% to 5% sporulation. As we classified 2-5% sporulation as susceptible and absence of *Vd3*, this plant would lack both *Vf* and *Vd3*. This is the only indication of a recombination between the *Vf*-locus and the *Vd3*-locus. The flanking markers confirm this recombination event around the *Vf* locus in this plant. This single plant is responsible for positioning *Vd3* just South of *Vf*, and not at the *Vf*-locus itself or North of *Vf*. If the sporulation of 2-5 % was regarded as resistant and presence of *Vd3* or all of the plants in class 1 and 2 were discarded, then *Vd3* would have been positioned in the same bin as the *Vf*-locus. We conclude there is evidence that *Vd3* is South of *Vf*, but for a firm proof additional observations are needed.

Patocchi et al. (1999a) during the fine mapping of the *Vf* gene classified about 9% of the plants as resistant but they showed the alleles in repulsion to *Vf* in the flanking markers. The exclusion of the phenotype data of these plants was necessary in the correct mapping of the gene as they demonstrated later with the map based cloning of the *Vf* gene (Patocchi et al. 1999b). In the mapping of the *Vbj* gene Gygax et al. (2004) found 13 resistant plants out of a population of 173 individuals, that did not have the marker alleles in coupling with the gene and 12 susceptible ones that had them. Finally Erdin et al. (2006) during the mapping of the *Vb* gene in the population 'Golden Delicious' x 'Hansen's baccata #2' identified six GPI plants. After the exclusion of the resistance data of these plants the tension that they observed in the map disappeared. Patocchi et al. (1999a) suggested two hypotheses to explain this situation; the first one is that these plants are double recombinants, and the second one that they were wrongly classified. As reported by these authors (Patocchi et al. 1999a; Gygax et al. 2004; Erdin et al. 2006), the presence of double recombinants is quite unlikely because of the distance of the flanking markers in coupling with *Vd3*. In our case we expect a frequency of double recombinants of 0.6 % and not the 3 % observed. The hypothesis of the wrong classification could be more probable supported by the fact that these plants were classified in the class 1 (one plant) and class 2 (two plants), which means from 1% to 5% of sporulation. Erdin et al (2006) suggested that the presence of susceptible plants showing the marker alleles in coupling with the resistance could be because of the presence of modifiers that might reduce the effect of the resistance gene.

The presence in a small genomic region of *Vd3*, the *Vf* locus composed by 4 R (resistance)-genes (*HcrVf*1, *HcrVf2*, *HcrVf3* and *HcrVf4*) (Vinatzer et al. 2001) and the *Vf2ARD* (Boudichevskaia et al. 2008) could indicate the presence of a gene cluster of *Vf*-like sequences. This is supported by the fact that Broggini et al. (2009) found two SSRs developed from BAC clones containing *Vf*-like sequences in the vicinity of the *Vf* locus. Another cluster of R-genes against *V. inaequalis* was found by Bus et al. (2004) in LG2. This cluster comprises four major genes as well as several race specific QTLs (Bus et al. 2004). This grouping of R-genes in clusters is found frequently in plants. The biological reason could be the generation of novel resistances through the unequal crossover between different genes (Hammond-Kosack and Jones 1997).

***Vd3* is a new apple scab resistance gene**

The genetic position of *Vd3* in LG1 permited us to show that *Vd3* is novel compared to all of the previously identified resistance genes against *V. inaequalis*, including *Vf*, *Va* and *Vf2ARD* genes (Patocchi et al. 1999a; Hemmat et al. 2003; Boudichevskaia et al. 2008). In the case of the *Vf* gene, the resistance spectra demonstrated that *Vd3* is not *Vf*. Whereas the *Vf* gene has a wide resistance spectrum conferring resistance to the *V. inaequalis* races 1 to 5 (Mc Hardy 1996), in our disease tests *Vd3* only confers resistance to the EU-NL-24 strain (race 7) capable of overcoming the resistance provided by *Vf* (Table 3).

Regarding the *Va* gene, although also mapped in LG1, the position is different to that one reported for *Vd3*. Gessler et al. (2006) reported the positions of the *Va* gene mapped by Zini (2005), who used the cultivar 'Freedom' as the resistance donor. This cultivar is carrying the *Vf* gene and another one coming from an unspecified 'Antonovka' clone. In the 2 locations reported the *Va* gene is outside the interval between the SSRs Hi12c02 and CH05g08 and in both cases at a distance of about 25cM from *Vf* (Zini 2005). This distance is in agreement to that one reported previously by Hemmat et al. (2003) using as resistance donor 'Antonovka PI 172633'. On the contrary, *Vd3* is close to *Vf* (Figure 2) and is within the region flanked by those SSRs. Using the RAPD marker P-136 described by Hemmat et al. (2003) no results were obtained (data not shown). The band of 700bp reported by these authors as linked to *Va* gene was not present in the population 2000-012C. Therefore, it was not possible to test the association between P-136 and *Vd3*. Moreover, the reaction types of both genes indicate that they are not the same, as *Va* induces a hypersensitive pit type reaction (Dayton and Williams 1968), whereas *Vd3* induces a chlorosis reaction too. Finally, taking into account the pedigree analysis showed in figure 3 we can say that the source of *Vd3* (D3) does not come from an Antonovka accession. The 129bp allele coming from D3

does not occur in the Antonovka accessions tested by Vinatzer et al. (2004) or in Antonovka 34.16 (unpublished data from HiDRAS database).

Recently Boudichevskaia et al. (2008) reported the mapping of sequences homologous to the *Vf* genes identified by Vinatzer et al. (2001) (*HcrVf* genes). The candidate gene *Vf2ARD*, developed on the basis of the divergences in the sequence of the C1 subdomain of the *HcrVf2* gene present in the apple accessions 'Antonovka', 'Realka' and 'Discovery', was mapped to the north of the *Vf* locus at a distance of 1.9 cM from the marker CH-*Vf1* (Boudichevskaia et al. 2008). Using the primers developed by these authors we mapped the *Vf2ARD* gene in a similar location but a distance between 2.5 and 3 cM depending on the map (Figure 2). So *Vd3*, that is south of the *Vf* locus, is located at a distance of about 5 cM from *Vf2ARD*. Moreover this marker was also present and mapped in the susceptible parent of our population (data not shown). So we can discard that *Vd3* and *Vf2ARD* were the same gene.

Bénaouf and Parisi (2000) detected in the cross 'Golden Delicuous'x *M. floribunda* 821 in addition to the *Vf*-resistance, another locus for resistance descending from *M. floribunda* 821. They named it *Vfh*, but they did not map it. According to Parisi et al. (2004) EU-NL-24 can sporulate on *M. floribunda* 821. This indicates that the *Vfh* gene is not the same as the *Vd3* gene.

Calenge et al. (2004) mapped QTLs for scab resistance using different strains of *V. inaequalis*. They used also EU-NL-24. The QTLs for EU-NL-24 were not mapped in the vicinity of the *Vf* locus in the progeny of the cross derived from 'Discovery', an English cultivar partially resistant to *V. inaequalis*, and 'TN10-8', a partially resistant hybrid derived from 'Schmidt's Antonovka PI 172632'.

In conclusion, we present in this work a new apple scab resistance gene that is only 1 cM south of the *Vf* locus. The *V. inaequalis* strain EU-NL-24 is virulent to *Vf*, but avirulent to *Vd3* cultivars. However, *Vd3* has not been effective against the majority of other *V. inaequalis* strains we used in our disease tests. The *Vd3* gene is south of the *Vf*-locus in contrast to previously mapped *HcrVf*-like sequences.

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Chapter 7

General Discussion

Apple (*Malus* x *domestica*) is a very important fruit crop across the entire world. Production of apples is continuously affected by many diseases and pests. Among them all, apple scab is a devastating fungal disease which causes severe damage to the crop and to apple production. This thesis describes molecular and cell biological techniques involved in developing genetically modified scab resistant apples with cisgenic and intragenic resistance genes. In this study, we have investigated the role of *HcrVf* genes (the only positionally cloned and published apple-derived scab resistance locus so far) in conferring scab resistance. Native regulatory elements (promoters and terminators) in two different lengths and the apple rubisco promoter and terminator were studied with respect to their effect on gene expression and on phenotype, i.e. resistance level, using a genetic modification system for production of selectable marker-free apple plants.

Genetically engineered crops and their acceptance

Apple scab is an economically devastating fungal disease caused by *Venturia inaequalis*. Most of the present day apple cultivars are susceptible to apple scab disease. Development of an apple cultivar resistant to apple scab through classical breeding takes many years due to long generation time and genetic drag. Therefore, development of cisgenic/intragenic apples resistant to apple scab is an attractive option. Many resistance genes have been mapped in apple, but only the *Vf* locus is widely used in apple breeding programs around the world and has recently been cloned. There are a number of apple cultivars available with *Vf* resistance, but new isolates have overcome the resistance offered by *Vf*. So to get durable resistance, it is essential to combine multiple, different resistance genes leading to gene pyramiding. Cisgenesis or intragenesis is the genetic modification approach of which the products can be considered comparable to the ones obtained by classical breeding. This approach can be used for gene pyramiding. Development of genetically modified crops can be categorized in three classes viz. transgenesis, intragenesis, and cisgenesis. Different approaches for development of cisgenic apples have been described in detail in Chapter 2 and the different steps leading to cisgenic apples as taken in our laboratory in the following chapters. In view of developing resistance to apple scab, cisgenesis and intragenesis approaches have been adopted. In case of cisgenic apples the *HcrVf2* gene is being used with its native promoter and terminator elements. In case of intragenic apples the *HcrVf2* coding sequence (the native gene does not contain any introns) has been combined with the apple rubisco promoter and terminator.

The term "cisgenesis" was first introduced in 1999 in a European project QLK5-CT-1999-01479 on sustainable production of transgenic strawberry plants, ethical consequences and potential effects on producers, environment and consumers by prof. Henk Jochemsen during one of the project meetings. Jan Schaart, who was involved in this EU project, devoted his PhD thesis on the development of cisgenic strawberries (Schaart 2004). The term "cisgenesis" was first published by Jochemsen (2000). Carl Nielsen was also member of that European project and introduced a few years later the term intragenic (Nielsen, 2003). This was followed in literature by several review articles on crop improvement using the plant's own DNA (Rommens 2004; Rommens et al. 2004). Conner et al. (2007) described intragenic vectors for gene transfer without making use of foreign DNA. They proposed that the resulting plants were non-transgenic, although developed by using the tools of molecular biology and plant transformation. The definition of cisgenesis was further specified and detailed by Schouten et al. (2006a).

The acceptance of genetically engineered foods by consumers is an important prerequisite for commercialization. This is especially important in the case of direct consumption of fresh products like fruits and vegetables. In a survey in the Netherlands only 4% of the consumers were willing to buy and eat apples, containing genes from micro-organisms or animals (Jan Gutteling, Pers. Comm.). In case genes would be introduced not from micro-organisms or animals but from a plant other than apple, the consumer preference to buy and eat increased to 20%, while 37% of the respondents were not willing to buy apples with genes from other plants (Jan Gutteling, Pers. Comm.). The willingness to buy and eat had risen to 35% when only apple genes were to be used for the genetic modification. When the consumers did receive additional information about the nature of and the reasons for genetic modification, the willingness to buy and eat apples with own genes only, increased to 47%. Lusk and Rozan (2006) showed similar improved willingness behavior of consumers. All these studies shifted the attention of scientists and breeders towards genetic modification using only the plant's own genes. This was further stimulated by the increasing availability of plant genes for all kinds of traits.

Considering that the products of cisgenesis resemble very closely plant products obtained by mutagenesis or traditional breeding, cisgenesis and the products thereof should proposed to be exempted from the GMO regulatory framework (Schouten et al. 2006a). The relevant position of gene integration in the genome through cisgenesis does not correspond to the natural position. But in natural situation also genes and other stretches of DNA may change position in the genome leading to translocations. In particular, cisgenic apple lines containing no selectable marker genes and only apple-derived scab resistance genes, such as *HcrVf2* and/or others, should be considered substantially equivalent to resistant cultivars

such as 'Santana' and should be allowed on the market under the same conditions as cv. 'Santana'.

Durable disease resistance

It took approximately 85 years to develop through classical breeding a cultivar like 'Santana', a resistant cultivar with the *Vf* locus against apple scab. After 4 to 5 years of its first introduction and cultivation of these resistant cultivars, the first reports were published about the evolution of two new virulent strains that can overcome the resistance of *Vf* (Parisi et al. 1993). To avoid resistance breakdown occurring so quickly, we should develop new strategies on producing more durable resistance using multiple genes. Durable scab resistance could be obtained by combining several independent major and/or minor genes resulting in a polygenic nature of the resistance. Bringing multiple genes together in a single elite cultivar by sexual crossing and selection is very time consuming in perennial crops such as apple mainly because the generation time is very long. The only option to obtain durable resistance by gene stacking in relatively short time is through cisgenesis/intragenesis. The identification and availability of molecular markers linked to the individual resistance genes *Va, Vh2, Vh4, Vbj, Vr2, Vd,* and *Vg* (reviewed by Gessler et al. 2006) can aid in subsequent gene isolation. Once isolated, they can be combined along with *Vf* to obtain more durable resistance.

Stacking multiple genes can be done in two ways (a) classical breeding which takes a lot of time and it is impossible to maintain the cultivar properties. The other option is by (b) genetic modification which takes less time compared to classical breeding and also the cultivar properties can be maintained. Stacking multiple gene through genetic modification can be performed in two ways. 1) Introducing all the available resistance genes in one go, i.e. cloned on a single T-DNA, into the cultivar of interest. Even though this method might seem to save a lot of time, obtaining and identifying a transformant with all the inserted genes intact and expressed at the desired level, might prove to be very difficult and time-consuming. Still, such plants can be obtained provided large number of transformants have been generated and can be analyzed for expression of all the inserted genes separately. Untergasser et al. (2010) transformed tobacoo and tomato with eight *Medicago truncatula* genes on a single T-DNA which are essential for *Rhizobium* symbiotic signaling and found that transgenic lines with variable level of expression of eight genes compared to wild type *M. truncatula*. 2) Introduction of one after the other when a new gene becomes available. This way, primary transformants carrying the first available resistance genes and expressing them leading to the desired phenotype, can be selected and cultivated, until a second series of resistance genes becomes available. Such proven valuable primary transformants can then be

subjected to a second transformation event receiving the new genes. Among the secondary transformants the best performing ones need to be identified again, i.e. selecting those individuals in which the secondary resistance gene functions at a similar proficient level as number one. This method requires a lot of time getting first good primary transformants, checking for expression of the inserted gene and then, using them for the second transformation with a second gene. After each time earlier transformed genes and new genes inserted due to new transformation events have to be analyzed for their expression. Even though one does not need to analyze equally large number of transformants as in method 1, the period required from transformation till getting confirmed transgenic line for each transformation event takes a long time and might be a critical factor. Chan et al. (2005) studied gene stacking in *Phalaenopsis* orchid for dual resistance against viral and bacterial pathogens through retransformation to the protocorm-like bodies (PLBs). Coat protein of Cymbidium mosaic virus (CymMV) was introduced into PLBs through particle bombardment and hygromycin was used to select putative transformants in the first transformation. They re-transformed primary transformants with pepper ferredoxin-like protein (*pflp*) which gives resistance against *Erwinia carotovora* subsp *carotovora* through *Agrobacterium*-mediated transformation in the second transformation. They added a culture of *E. carotovora* to an antibiotic-free plate which contained the transformed PLBs, for selection of resistant secondary transformants. So, they used *E. carotovora* as a non-antibiotic selection agent, thus screening simultaneously for proficient expression levels of the second gene-of-interest. So keeping time as a critical factor, it is better to transfer all the genes at once with all genes in one binary construct. In stacking of genes using one T-DNA, no limit to the number of genes to be introduced has been determined, yet. Eight genes, each of which 10 kb in length, were inserted in one binary construct and transferred successfully into tobacco (Untergasser et al. 2010).

Introduction of multiple genes in one go requires only one transformation protocol which is based on one selectable marker system. But in re-transformation multiple selection systems are needed, or the selectable marker has to be removed prior to subsequent secondary transformations.

Scab-resistant apples can be developed by transforming *HcrVf2* either with its own short promoter of 288 bp of length (cisgenic) or with the rubisco promoter (intragenic) using marker-free systems. More durable resistance can be obtained by stacking more cisgenes or intragenes that are presently being identified (Chapter 6) and isolated. Both mentioned approaches, in one go or one after the other, can be used in apple.

Generating marker free plants

Marker-free plants can be obtained in two ways, firstly by introducing genes of interest without using selectable marker genes (Malnoy et al. 2010). Every regenerant plant has to be checked individually by PCR for the presence of the inserted gene-of-interest. This can be quite laborious. Secondly, the selection marker can be excised after selection and testing of the transformant. In this chapter we will present the first results of marker gene removal in order to obtain marker-free apple transformants.

There are different systems using recombination/excision to remove selection markers and any other undesired DNA sequences leading to a marker-free, cisgenic/intragenic plant, namely Cre/Lox, FLP/FRT, R/Rs. In our laboratory, a binary vector, pMF1, was developed which is based on the R/Rs system for marker removal (Figure 1.) (Schaart et al. 2010a). The selection marker and reporter genes can be placed in between the recombination sites (Rs). R/Rs uses the recombinase gene R from *Zygosaccharomyces rouxii* to excise the DNA fragment between the Rs. After producing the cisgenic/intragenic plants in this way, the absence of the DNA fragment which is in between the Rs should be verified. The R/Rs system is currently being tested in crops such as apple, potato, strawberry, clover and tobacco. Some preliminary results are presented here that were obtained in the development of marker-free transgenic apple plants. A type 1 α-hordothionin (*hth*) gene from barley under regulation of the chrysanthemum rubisco small subunit promoter (P_{CmRbc}) and terminator was introduced into scab-susceptible cultivars 'Gala' and 'Elstar' through *Agrobacterium tumefaciens*-mediated transformation. The transgenic tissues carrying an *npt*II selectable marker gene were selected on medium containing kanamycin. The selection marker in these transformants was subsequently excised using the R/Rs system. Dexamethasone (Dex), a glucocorticoid hormone was used to induce activity of the recombinase R, which excises the fragment between the Rs. The site-specific Recombinase (R) and the recombination sites (Rs) are from the yeast *Z. rouxii*. These recombination sites can be between 59-82 bp in length and consist of three direct imperfect 12 bp repeats and one inverted repeat. For recombination to occur, two Rs should be in the same orientation. The Ligand Binding domain (LBD) of rat glucocorticoid receptor was translationally fused to the C-terminus of the R gene. In the plant cell this prevents movement of the enzyme into the nucleus and hereby prevents excision. Movement into the nucleus and thus recombination activity is restored upon addition of Dex. The pMF1 binary vector system further makes use of a negative selectable marker, the *codA* gene, which is also placed between the Rs. *CodA* is derived from *E. coli* and converts the non-toxic compound 5-fluorocytosine (5-FC) to toxic 5-fluorouracil (5-FU). If after recombination the *codA* gene is (still) present in transgenic plants, 5-FC added to the medium will be converted to 5-FU and those cells cannot survive. However, if the *codA* is completely excised, the marker-free plants can grow on medium

supplemented with 5-FC. In pMF1, *codA-npt*II, a hybrid gene combining both positive (*npt*II) as well as negative (*codA*) selection is employed.

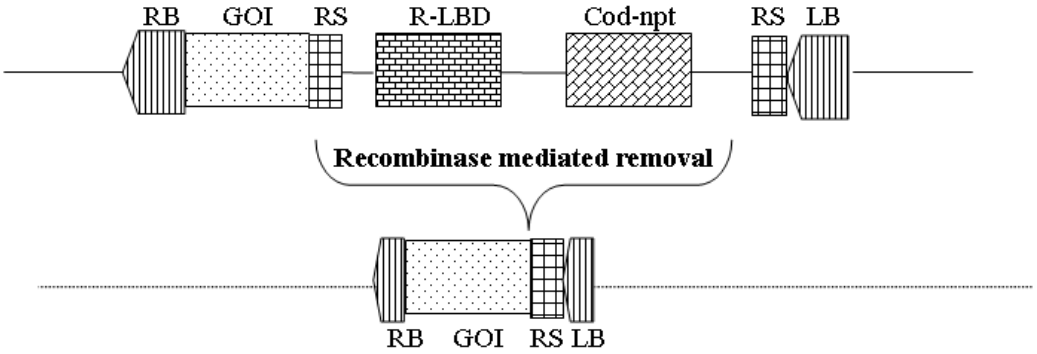

Figure 1. T-DNA of the pMF1 marker-free vector before and after recombination induced by dexamethasone treatment**.** RB and LB, Right Border and Left Border; GOI, Gene-of-Interest; Rs, Recombination sites; R-LBD, Recombinase and ligand binding domain; Cod-npt, *codA* and *nptII* bifunctional selection marker

In total, 21 apple transformants carrying as gene-of-interest the aforementioned barley *hth*-gene in the pMF1 vector as shown in Figure 1 were selected for Dex treatment. Nine of them had a single copy insertion and eight were having multiple copy insertions. Of the nine single copy insert lines, five did not give regeneration on 5-FC medium and 4 did. Similarly of the eight multiple copy insert lines, one did not give regeneration on 5-FC medium and 7 did.

Table 1. T-DNA copy number in transgenic lines and the percentage of MF plants among 10 randomly chosen regenerants (derivatives) after Dex treatment.

Transgenic line	Copy number	Percent MF plants	Derivative
3G22	1	90	3G22-11
			3G22-14
			3G22-15
			3G22-16
			3G22-17
			3G22-19
3G37	2	60	3G37-21
			3G37-22
			3G37-25
			3G37-27
			3G37-28
3G3	3	90	3G3-41
			3G3-43
3G30	2	100	3G30-101
			3G30-107
			3G30-110

The derivatives given in this table represent the individuals that were incorporated in Southern analysis (Figure 2).

Among the 11 regenerating lines, variation was observed for the percentage of marker-free plants that were obtained after the Dex treatment, as determined by PCR molecular analysis. They were categorized into three groups namely 1) 0-20% MF plants which has 5 lines, 2) 60% MF plants which has 1 line, 3) 90-100% MF plants which has 5 lines. Southern analysis was performed to study the number of the T-DNA inserts and, later, the occurrence of chromosomal re-arrangements using primary transgenic lines 3G22, 3G37, 3G3, and 3G30 along with their respective putative MF derivatives (Table 1 and Figure 2).

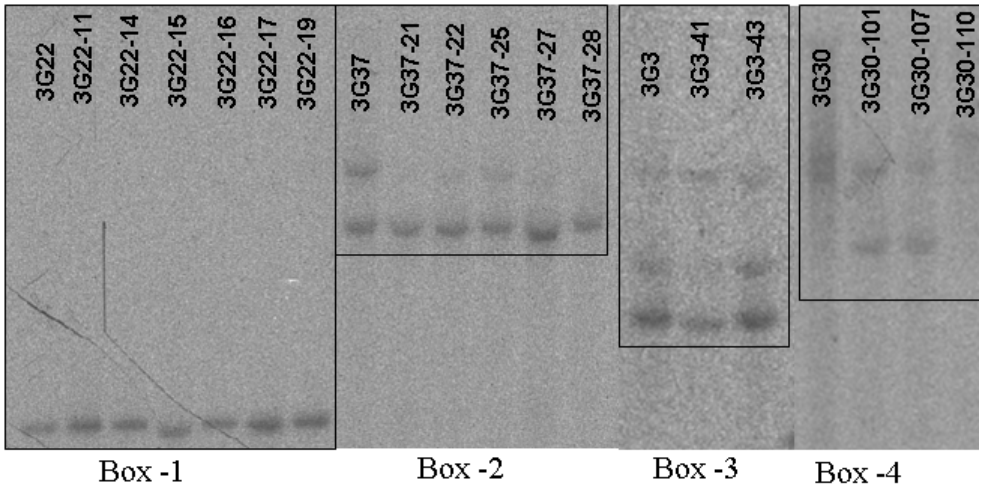

Figure 2. Southern blot showing T-DNA stability after Dex treatment and excision by recombination: Box 1 and 3, no change in T-DNA integration pattern; boxes 2 and 4, changes indicative for putative chromosomal re-arrangements; probe: *hth* ; DNA digested by *BglII*

In box-1 we can see clearly line 3G22 having a single transgene insertion and its derivatives after Dex treatment showing the same fragment when probed with *hth*. Probing with *npt*II gave no hybridization and no visible fragments. Together with the negative PCR results with *npt*II and LBD primers on the derivatives, this provided evidence for clear excision of the selection marker resulting in marker-free plants with no indication for chromosomal re-arrangements. In box-2 3G37, which has two transgene insertions, and its derivatives after Dex treatment are shown. Here, in three of the five derivatives a reduction was observed in the number of fragments, from two to one, with the remaining fragment being identical to the one present in the untreated line 3G37. In the two other derivatives the upper band seems to be less prominent. These phenomena can be explained by a two copy insert in a head-to-tail configuration and by non-complete excision (Figure 3). The Southern data suggest the occurrence of recombination between multiple Rs sites in a two- or three-step process (e.g. between sites 1 and 3 and subsequently between the new one and 4 or between 1 & 2 and 3 & 4 [case 2] and subsequently between the two new ones), or in a single step (between 1 & 4), all leading to the situation with one copy left in the same chromosomal location. Cells that did not carry out the recombinations gave rise to the vague upper, original band. This might be an indication for chimerism for the recombination/excision event. However, all derivatives proved to be marker-free by PCR

analysis and Southern analysis with *npt*II as probe, so perhaps we need to look further for explanations.

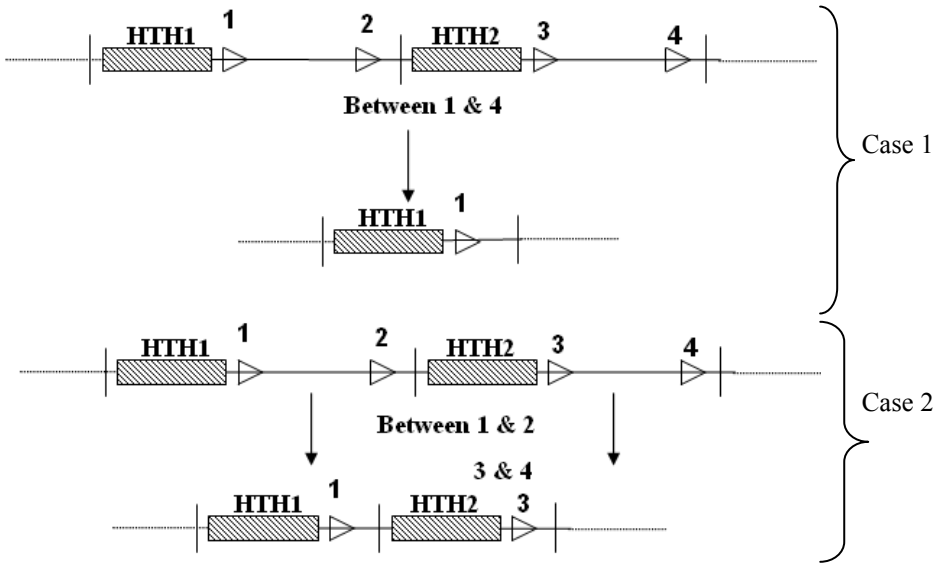

Figure 3. Possible chromosomal re-arrangements in 3G37 derivatives after Dex treatment assuming a head-to-tail configuration of the two inserted T-DNA copies in 3G37. **HTH1** and **HTH2-** Hordothionin first and second copy; 1, 2, 3 and 4 are recombination sites

In box-3 we can see line 3G3 with three transgene insertions and its derivatives after Dex treatment, 3G3-41 and 3G3-43. There are no changes in the patterns, leading to the conclusion that here, also in a multiple copy insert situation, the appropriate intended Rs were used and clear excision of selection markers was possible without leading to chromosomal re-arrangement. Again probing with *npt*II and the PCR reactions with the earlier mentioned primers were negative in the derivatives backing up the conclusion on being marker-free. Finally, in box-4 3G30 was found to contain two transgene insertions and its derivatives 3G30-101 and 3G30-107 showed two different transgene insertions, and 3G30-110 showed only one band at a different position from any of the bands in 3G30 or the other derivatives. This can be regarded as a clear indication for the occurrence of chromosomal re-arrangements.

So we can conclude from the preliminary experimental results presented above, that marker-free plants can be obtained using the pMF1-based system, both in case of single copy inserts as well as in case of multiple T-DNA inserts without any chromosomal re-

arrangements. However, chromosomal re-arrangements were detected, especially in cases with multiple copy inserts. The precise nature of these changes needs to be studied further by checking the flanking sequences of the T-DNAs of transformants before and after Dex treatment. This illustrates the need to characterize thoroughly all marker-free plants after Dex treatment, not only by PCR but also by Southern analysis. It also means that after retransformation using the pMF1 marker-free system, thus introducing at least one secondary T-DNA carrying two Rs into a primary marker-free transformant still containing one Rs copy, one has to be aware that in this situation with three Rs undesired chromosomal rearrangements might take place after the second Dex treatment. Careful checking the final plant products remains compulsory.

Four *HcrVf* transformants, SP*HcrVf2*-11, LP*HcrVf2*-4, P_{MdRbc}*HcrVf2*-12 and P_{MdRbc}*HcrVf2*-11, were chosen for Dex treatment to make them marker free and two of them reached the regeneration stage.

Resistance genes

In plants the resistance gene (*R* gene) will interact with the corresponding avirulence gene (*Avr* gene) of the pathogen which is popularly known as the "gene-for-gene" concept (Flor 1971). The *R* genes were broadly classified into five classes based on structure and domains (reviewed in Lehmann 2002). These five different *R*-gene classes are presented in Figure 4.

Lehmann (2002) reviewed R genes and their domains as follows:

1) Nucleotide binding sites-leucine rich repeats (**NBS-LRRs)** genes constitute the largest family among R genes in plants. There are two major NBS-LRRs categories namely
   A) Toll and Interleukin-1 receptor-NBS-LRRs (**TIR-NBS-LRRs)** E.g. *RPP5, N*
   B) Coiled coil-NBS-LRRs (**CC-NBS-LRRs)** E.g. *RPS2, RPM1*
2) Extracellular leucine rich repeats-transmembrane (**LRRs-TM)**-also known as RLPs. E.g. *Cf-2, Cf-4, Cf-9*
3) Extracellular leucine rich repeats-transmembrane-kinase (**LRR-TM-kinase**)-also known as RLKs. E.g. *Xa21*
4) Intracellular Serine/threonine protein kinase E.g. *Pto*
5) Detoxifying enzyme HC-toxin reductase. E.g. *Hm1*

Extracellular LRRs and *HcrVf* genes

Extracellular Leucine Rich Repeats (eLRRs) were first discovered in leucine-rich α2-glycoprotein in human serum (Takahashi et al. 1985). Plant cell receptors contain LRRs as extracellular proteins and they are typically 20-29 amino acid residues sequence motifs with a number of functions such as plant disease resistance, regulation of gene expression, cell polarization (Kobe and Kajava 2001). The plant specific eLRR motif contains about 23 to 25 amino acids and a conserved consensus sequence as LxxLxxLxLxxNxLT/SGxIPxxLGx (Federici et al. 2006) where L is leucine, x is any amino acid, N is asparagine, T is threonine, S is serine, G is glycine, I is isoleucine, and P is proline.

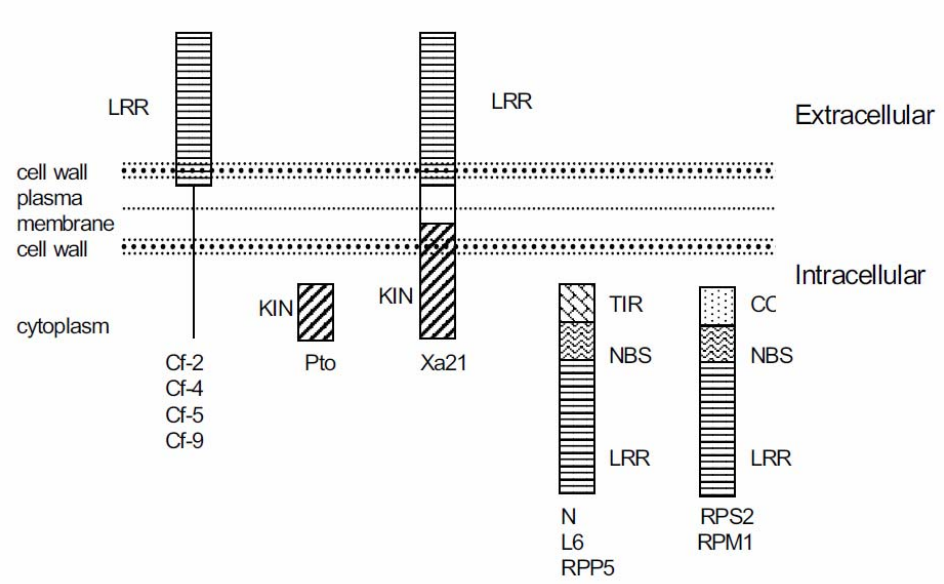

Figure 4. Representation of location and structure of the five main classes of plant disease resistance proteins. Examples of genes are mentioned below each class. LRR, leucine-rich repeat; NBS, nucleotide binding site; KIN; ser/thr protein kinase; CC, coiled coil domain; TIR, toll and interleukin receptor domain (Adapted from Lehmann 2002).

The largest group of eLRR proteins consist of receptor-like kinases (RLKs) which contain an eLRR domain, a transmembrane domain and a cytoplasmic kinase domain. The second largest group consists of receptor like proteins (RLPs) which contain an eLRR domain and a transmembrane domain only (Wang et al. 2008). RLPs were proven to have a role in disease resistance, e.g. *Cf* and *Ve* proteins in tomato against *Cladosporium fulvum* and *Verticillium* spp. respectively, and *HcrVf2* in apple against *Venturia inaequalis* (Wang et al. 2008). These RLPs have varying number of extracellular leucine rich repeat (eLRR)

domains, 25-38 in *Cf* genes (Dixon et al. 1998) and 26-30 in *HcrVf* genes (Xu and Korban 2002).

An example of an RLK is *FLS2*, which recognizes flagellin from bacteria and leads to activation of downstream signaling such as generation of ROS, MAP kinase activation, ethylene production and induction of gene transcription (Bar and Avni 2009b). As mentioned earlier, RLPs lack the kinase domain which has an important role in intracellular signaling (Joosten and de Wit 1999). There is a hypothesis that other proteins might play a role in the entire process between the perception of the pathogen and the interaction between R/AVR genes and the downstream signaling leading to the actual plant defense responses (Rivas and Thomas 2005). An example of such a role for other proteins is given by Bar and Avni (2009a). Ethylene-inducing-xylanase (EIX) is a fungal protein elicitor inducing ethylene biosynthesis, expression of PR proteins, and the hypersensitive response (HR). *LeEix2* (in *LeEix* locus of tomato) is a LRR-RLP. They showed that other proteins such as AP2 and EHD2 are involved in signaling the defense response through the interaction of *LeEix2*-AP2-EHD2-EIX (Bar and Avni (2009b). Bar and Avni (2009c) investigated and concluded that the coiled coil domain of EHD2 is crucial for binding of EHD2 to the *LeEix2* and also to inhibit endocytosis in plants.

Another example of involvement of other proteins in defence related signaling can be explained by another *Cf-2* gene in tomato. *Cf-2* is an RLP which gives resistance against *C. fulvum* in tomato. Wulff et al. (2009) described *Cf2-Rcr3-Avr2* interaction. They stated that recognition of the Avr2 effector protein of *C. fulvum* by the *Cf-2* gene is through tomato *Rcr3* which is a secreted cysteine protease (Krüger et al. 2002). *Avr2* is a cysteine protease inhibitor which binds and inhibits *Rcr3* and thus *Rcr3-Avr2* complex enables the Cf-2 protein to activate an HR (Rooney et al. 2005).

Other possible descriptions about the involvement of transmembrane domain (TM) in the singalling in RLPs have been reported below. A conserved motif in the TM GxxxG of RLPs is likely involved in the protein-protein interaction. This motif could aid in dimerization and activation as was found in case of *ErbB2*, a mammalian receptor kinase (Bennasroune et al. 2004; Fritz-Laylin et al. 2005). This conserved motif in RLPs may play an important role in mediating interactions between RLPs or between RLPs and other transmembrane proteins (TM) and thus be involved in RLP signal transduction (Fritz-Laylin et al. 2005).

HcrVf (Homologous *Cladosporium fulvum* resistance genes of *Vf* region) genes are similar to *Cladosporium fulvum* (*Cf*) resistance genes of tomato which belong to the eLRR (extra cellular LRR and transmembrane domains) class of R genes. The deduced amino acid

sequences of *HcrVf* genes showed very high similarity to the *Cf-9* gene of tomato (Vinatzer et al. 2001).

Isolation of *HcrVf1* and *HcrVf2* which are RLPs and their role in conferring resistance to apple scab have been described in Chapter 3 and Chapter 4, respectively.

Induction of *HcrVf* gene

HcrVf genes are thought to be receptors and as such they should be present even before pathogen attack (Szankowski et al. 2009). The resistance assay results have been described and discussed in the Chapter 4. In a preliminary experiment, we inoculated EU-B05, a *Vf* avirulent strain of *V. inaequalis*, to study the induction of *HcrVf2* gene expression in cv. 'Santana'. The top two leaves of the plants were used for inoculation. In this pilot experiment, samples were harvested prior to and 5-days after inoculation in order to determine changes in expression levels of *HcrVf2*. The induction was studied through quantitative RT-PCR (qRT-PCR). The expression of *HcrVf2* was normalized with the expression of actin (reference gene). The expression of *HcrVf2,* five days after inoculation, was found to have increased to a level of about 187 fold higher when compared to the basal level (Figure 5).

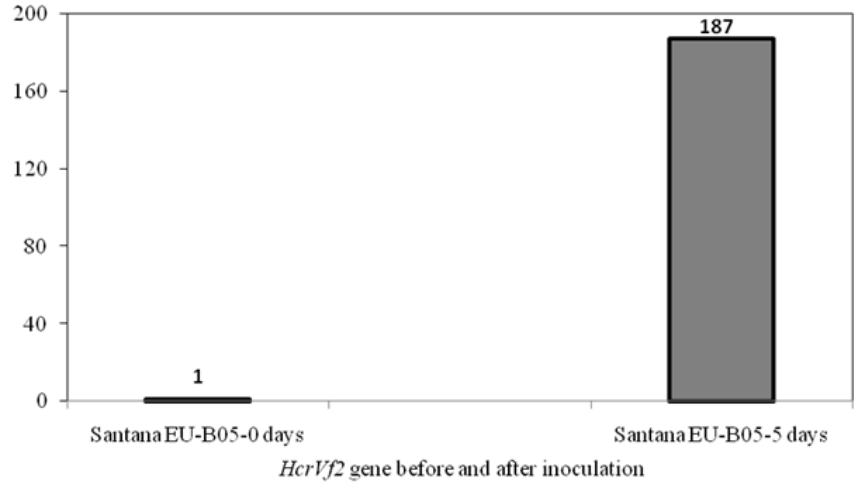

Figure. 5. Induction of *HcrVf2* gene in Santana. S-EU-B05-0 days – Expression in 'Santana' before inoculation with *Vf* avirulent isolate EU-B-05; S-EU-B05-5 days – Expression in 'Santana' 5 days after inoculation with *Vf* avirulent isolate EU-B05.

Earlier, Malnoy et al. (2008) inserted *Vfa1* (which is identical to *HcrVf1*) and *Vfa2* (which is identical to *HcrVf2*) into cvs. 'Galaxy' and 'McIntosh'. They observed an induction of 5 to 10 fold and 2 to 8 fold for *HcrVf1* expression in cvs. 'Galaxy' and 'McIntosh' respectively compared to the control plants and they showed an increase of 4 to 30 fold and 5 to 8 fold for *HcrVf2* expression in cvs. 'Galaxy' and 'McIntosh' respectively compared to the control plants 24 hours after inoculation. In the preliminary induction experiments we observed that the upregulation as found in cv. 'Santana' 5-days after inoculation with the fungus, could not be observed in two of the transformants that we tested, SP*HcrVf2*-15 and LP*HcrVf2*-16. Interestingly, these plants showed sporulation when checked for resistance in the greenhouse with scab, at levels similar to those observed in cv. 'Gala' (susceptible control) (result not shown). These data together suggested that basal expression of the active *HcrVf2* gene at the level of unchallenged cv. 'Santana', as seen in SP*HcrVf2*-15 and LP*HcrVf2*-16, is not enough to give resistance, but that expression must be induced. Assuming the *HcrVf2* protein functions as a receptor, it is either not functioning properly in these two lines in perceiving the signal molecules from the pathogen or it is in some way obstructed in boosting its own expression as a response. As a consequence, the signal might not be properly transduced leading to sporulation. The generation of a specific amount of *HcrVf2* protein molecules is apparently essential for providing an efficient response as defense. In conclusion, our preliminary data indicated that upregulation after inoculation might be essential for good resistance. Based on the all the above investigations with respect to *Cf-2* and other RLPs, we can predict that other proteins are also involved in the signaling, the defense response and further research is needed in this aspect.

In the previous expression studies we have demonstrated the presence of very high expression levels (57 to163 fold in transformants compared to cv. 'Santana') of *HcrVf2* when regulated by the apple rubisco promoter and terminator (Chapter 3). Since rubisco is light induced and may not play any role in defense against pathogens, most likely in these transformants there will be no inoculation-induced upregulation of *HcrVf2* expression. However, the basal level of *HcrVf2* expression is already high in P_{MdRbc}*HcrVf2* transformants and apparently sufficient to give a high level of scab resistance. Further experiments in apple are necessary to study the process of pathogen identification, initiation of signal transduction pathways leading to upregulation of defense genes.

In conclusion, consumers prefer GM apples that have received genes from apple itself. In addition, durable or sustainable cultivation is highly appreciated, not only by consumers but also by growers. For this, it is essential that multiple apple derived resistance

genes are identified and combined into the consumer's favorite elite cultivars. These combinations can be made prior to transformation or with several retransformations. Marker-free systems are a prerequisite in the last case but also for the generation of cisgenic or intragenic plants devoid of any undesired selectable marker genes.

The first results of the recombination-based marker-free system in developing cisgenic and intragenic apple plants have been obtained in our laboratory after Dex treatment to excise selection markers in lines having obtained single or multiple T-DNA inserts. The lines SP*HcrVf2*-11 and P_{MdRbc}*HcrVf2*-11 have been used to excise the selection marker and to regenerate cisgenic and intragenic apple plants, respectively, and those will be tested in the field. With respect to gene pyramiding, the transformant lines with multiple copy inserts can give an indication as to what can be expected after Dex treatment at the level of genome integrity. They proved promising, but chromosomal re-arrangements could occur and should be checked for. Finally, it was found that the mere presence of the *HcrVf2* gene and basal expression is not enough for resistance; induction of expression to high levels is essential. In case of the rubisco promoter these levels can be reached without induction and this might explain the effectivity of these constructs.

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Summary

Apple (*Malus* x *domestica*) is one of the important fruit crops of the world. It is mainly cultivated in temperate regions. Apple fruit contains many health beneficial compounds which may play an important role in reducing cancer cell proliferation and lowering the level of cholesterol.

Apple production can suffer from several pests and diseases and among them scab is very important. Apple scab is a fungal disease caused by *Venturia inaequalis*. The pathogen is a facultative saprophyte that grows during the growing season subcuticularly on the host. Most of the present day high quality apple cultivars are susceptible to apple scab. The crop loss due to apple scab has been amount to more than 70%. Fruit growers usually spray fungicides 15 times or more in a season to control the scab disease. To reduce the use of chemicals, it is absolute necessary to develop apple varieties with durable scab resistance.

Conventional breeding in apple has some drawbacks such as long generation period, genetic drag and the self-incompatible sexual reproduction system. Therefore, stacking of more than one resistance gene by classical introgression breeding is inefficient. Genetic modification is an alternative option to improve the existing scab-susceptible varieties into scab-resistant ones. However, consumer acceptance of transgenic food in Europe is a problem. Therefore, we developed a genetic modification system with cisgenes and intragenes instead of transgenes. Cisgenes are genes from the plant itself or from crossable species with their natural introns and own regulatory elements in normal sense orientation. Intragenes are like cisgenes containing only functional parts of genes from the plant itself or from crossable species, however, these functional parts originate from different genes. All these genes or gene parts are belonging to the normal breeder's gene pool. Transgenes are synthetic genes or (partly) origination from non-crossable species, like viruses and microorganisms. Transgenes are representing a new gene pool for plant breeding. GMO-regulations have been developed for transgenes. Societal research showed that consumer preference for cisgenic food is higher than for transgenic food. Cisgenic or intragenic plants can be developed by transferring the desired scab resistant genes into the scab-susceptible cultivar through *Agrobacterium tumefaciens*-mediated transformation. Transformation aimed at cisgenesis or intragenesis should be done either without the use of selectable marker genes or by using selection markers first and eliminating them subsequently after selection of transformants. In this thesis almost all steps have been made to come to cisgenic apple plants with resistance to scab disease (chapter 2).

Although many scab resistance genes have been identified and mapped, only *Vf* has been positionally cloned. *Vf* is a locus with four paralogs namely *HcrVf1* (**H**omologues of ***C****ladosporium fulvum* **r**esistance genes of ***Vf*** region), *HcrVf2*, *HcrVf3*, and *HcrVf4*. Only *HcrVf1* and *HcrVf2* are considered as being functional. In conventional breeding *Vf* inherits as a single locus so it is not possible to study the individual role of *HcrVf1* and *HcrVf2* in conferring resistance against scab using conventionally bred material. The present study was set up to study in depth the roles of *HcrVf1* and *HcrVf2* separately in conferring resistance to apple scab, using *A. tumefaciens* mediated transformation. Both isolated genes were regulated as cisgenes by their own promoter and terminator sequences. The two cisgenes were used in two different lengths of the 5'-upstream sequences, so with a short promoter (SP) and a long promoter (LP) i.e. 312 bp and 1990 bp for *HcrVf1* and 288 bp and 2000 bp for *HcrVf2*. *HcrVf1* and *HcrVf2* were also combined with the apple rubisco promoter and terminator into intragenes because these regulatory elements were found to give high expression in plants. The *HcrVf1* and *HcrVf2* cisgenes and intragenes were inserted into the susceptible cv. 'Gala', using the marker free system pMF1. Several apple transformants were selected for further characterization.

Micrografting was carried out in order to take the 'in vitro' transformants to the greenhouse. This method proved to promote growth better than rooting of 'in vitro' transformants. Apple transformant 'in vitro' shoots were used as scions and grafted onto the apple seedling rootstocks. Micrografts were ready for further testing 4 to 5 weeks after grafting. At this stage the young leaves were collected for isolation of DNA and RNA. Southern hybridization was performed to check the inserted T-DNA copy number. For this, the selection marker gene *npt*II was used as a probe. Most of the transformants (17) were found to have a single T-DNA insert and seven transformants showed two T-DNA inserts. Subsequently, *HcrVf* gene expression in transformed lines was studied through quantitative RT-PCR (qRT-PCR) in relation to the natural *HcrVf* expression in the resistant cv. 'Santana'. In case of *HcrVf1* transformants, expression by LP was significantly higher than by SP, while in *HcrVf2* transformants no significant difference between SP and LP could be demonstrated. Both *HcrVf1* and *HcrVf2* genes showed highest expression when regulated by the apple rubisco promoter and terminator. Two *HcrVf2* transformants, LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12, showed the highest gene expression for the cisgene and intragene situation, respectively. Among *HcrVf* transformants, no significant correlation was observed between inserted gene copy number and gene expression level (Chapter 3).

Micrografted cvs. 'Santana' (resistant control containing *Vf* through classical breeding), 'Gala' (susceptible control) and different micrografted apple transformants were tested for scab resistance against *V. inaequalis* isolate EU-B05. The top four leaves were

used for inoculation with *V. inaequalis*. Seventeen days after inoculation, the plants were scored for sporulation using a quantitative scale. All the *HcrVf1* transformants showed complete sporulation similar to the level in cv. 'Gala', indicating that *HcrVf1* is not giving resistance. On the other hand, 10 out of the 13 *HcrVf2* transformants showed resistance at levels that were statistically similar to cv. 'Santana'. Two *HcrVf2* transformants, LP*HcrVf2*-4 and P_{MdRbc}*HcrVf2*-12, showed the best resistance. A negative correlation between *HcrVf2* gene expression and sporulation was observed i.e. as gene expression increased there was a decrease in the fungal sporulation (Chapter 4).

The results obtained by the scab experiment were used to select *HcrVf1* and *HcrVf2* transformants to check the resistance spectrum against different isolates of *V. inaequalis*. The plants were inoculated with four avirulent isolates of the pathogen and two isolates virulent to the resistant cv. 'Santana'. The top two leaves were inoculated with fungal spores and the inoculated plants were scored for sporulation 21 days after inoculation. All the *HcrVf1* transformants showed heavy sporulation of all the isolates used and they were behaving like untransformed cv. 'Gala'. The *HcrVf2* transformants were behaving like cv. 'Santana' indicating that the resistance coming from the *Vf* gene cluster is from *HcrVf2* alone (Chapter 5).

In order to increase the durability of resistance against scab, it is desired to stack several resistance genes into apple cultivars either by classical breeding or by genetic modification. To use it in a cisgenic or intragenic approach, new scab resistance genes have to be identified in apple and cloned. In chapter 6 it is described how a novel scab resistance gene, *Vd3*, has been identified and genetically mapped in the resistant selection "1980-015-025". In the study we used the F_1 progeny 2000-012 that is derived from the crossing between the resistant parent 1980-015-025 and the susceptible parent 1973-001-041. Mainly DArT markers were used in this genetic mapping study. Other known markers, such as SSRs, P-136 (RAPD marker), and *Vf2ARD* (RGA marker), were used for annotation of the linkage groups. The *Vd3* gene has been mapped 1 cM to the south of the *Vf* gene cluster in repulsion phase on linkage group 1. Paternity tests have indicated that clone 1980-015-025 has inherited the *Vd3* gene from founder accession D3. This gene can provide resistance against the virulent isolate EU-NL24, which can overcome the resistance of *Vf* and *Vg*. However, this gene cannot provide resistance against other isolates (Chapter 6).

The results described in this thesis are of practical importance. Cisgenesis or intragenesis can be employed to provide multiple gene resistance against scab in apple without linkage drag problems as observed during classical introgression breeding. Our first potential cisgenic scab resistant 'Gala' plants with the *HcrVf2* gene are being developed which can be used in regions free of virulent isolates. The cisgenic approach is essential in

rapid improving a crop such as apple where it takes many decades through conventional breeding.

Samenvatting

Appel (*Malus x domestica*) is een belangrijk fruitgewas in de wereld. Het wordt voornamelijk geteeld in gebieden met een gematigd klimaat met wat vorst in de winter. Appels bevatten veel gezondheidsbevorderende stoffen die een belangrijke rol kunnen spelen bij het tegengaan van de ontwikkeling van kankercellen en bij de verlaging van het cholesterolniveau.

De productie van appel kan te lijden hebben van diverse plagen en ziekten waarvan schurft een erg belangrijke is. Appelschurft is een schimmelziekte die veroorzaakt wordt door *Venturia inaequalis*. Dit pathogeen is een facultatief saprofyt die gedurende het groeiseizoen subcuticulair op z'n gastheer groeit. De meeste huidige elite appelrassen zijn vatbaar voor appelschurft. De oogstverliezen veroorzaakt door appelschurft kunnen oplopen tot meer dan 70%. Fruittelers spuiten normaal gesproken 15 keer of vaker per seizoen met fungiciden om appelschurft te bestrijden. Om het gebruik van chemicaliën terug te dringen is het absoluut noodzakelijk om appelrassen met een duurzame schurftresistentie te ontwikkelen.

Conventionele veredeling van appel kampt met een aantal bezwaren zoals een lange generatie tijd, genetische 'drag' en zelf-incompatibiliteit bij geslachtelijk voortplanting. Daardoor verloopt het stapelen van meer dan één resistentiegen via klassieke introgressieveredeling inefficiënt. Genetische modificatie is een alternatieve mogelijkheid om bestaande schurftgevoelige rassen om te zetten in schurftresistente rassen. In Europa is de acceptatie van transgeen voedsel door consumenten echter een probleem. Om die reden hebben we een genetisch modificatie systeem ontwikkeld met cisgenen en intragenen in plaats van transgenen. Cisgenen zijn genen afkomstig van de plant zelf of van kruisbare verwanten met hun eigen natuurlijke intronen en regulerende elementen in een normale 'sense' oriëntatie. Net als cisgenen bevatten intragenen functionele elementen van genen van de plant zelf of van kruisbare soorten, maar de functionele onderdelen zijn afkomstig van verschillende genen. Al deze genen of onderdelen van genen behoren tot de gebruikelijke genenbron van de veredelaar. Transgenen zijn synthetische genen of (deels) afkomstig van niet-kruisbare soorten, zoals virussen en micro-organismen. Transgenen vertegenwoordigen een nieuwe genenbron in de plantenveredeling. Regelgeving mbt genetisch gemodificeerde organismen is voor transgenen ontwikkeld. Sociologisch onderzoek laat zien dat consumenten de voorkeur geven aan cisgeen voedsel boven transgeen voedsel. Cisgene of intragene planten kunnen ontwikkeld worden door (het) gewenste schurftresistentiegen(en) naar schurft-vatbare rassen over te brengen door middel van transformatie met behulp van

Agrobacterium tumefaciens. Transformatie gericht op cisgenese of intragenese moet worden verricht òf zonder gebruik te maken van selectiegenen òf door in eerste instantie selectiegenen te gebruiken en ze daarna, wanneer transformanten geselecteerd zijn, weer te verwijderen. In dit proefschrift worden bijna alle stappen gezet om tot cisgene appelplanten met resistentie tegen schurft te komen (Chapter 2).

Ondanks het feit dat veel schurftresistentiegenen zijn geïdentificeerd en in kaart gebracht, is alleen *Vf* moleculair geïsoleerd. *Vf* is een locus met vier paralogen, namelijk *HcrVf1* (**H**omologen van ***C****ladosporium fulvum* **r**esistentiegenen van het ***Vf*** gebied), *HcrVf2*, *HcrVf3* en *HcrVf4*. Alleen *HcrVf1* en *HcrVf2* worden als functioneel beschouwd. In conventionele veredeling erft *HcrVf* als een enkel locus over en daarom is het niet mogelijk om met conventioneel veredelingsmateriaal de afzonderlijke rol van *HcrVf1* en *HcrVf2* in het bewerkstelligen van resistentie tegen schurft te onderzoeken. Het beschreven promotieonderzoek is opgezet om, gebruik makend van transformatie met behulp van *Agrobacterium tumefaciens*, de afzonderlijke rol van *HcrVf1* and *HcrVf2* in het bewerkstelligen van resistentie bij appel te bepalen. Beide geïsoleerde genen werden als cisgenen gereguleerd door hun eigen promotor en terminator sequenties. Beide cisgenen werden gebruikt met twee verschillende lengtes van hun 5'-stroomopwaarts sequenties, dus met een korte promotor (SP, short promotor) en een lange promotor (LP) van 312 bp en 1990 bp voor *HcrVf1* en 288 bp en 2000 bp voor *HcrVf2*. *HcrVf1* en *HcrVf2* werden ook gecombineerd met de appel rubisco promotor en terminator tot intragenen, omdat bekend is dat deze regulerende elementen een hoge expressie in de plant geven. De *HcrVf1* en *HcrVf2* cisgenen en intragenen werden in het vatbare ras cv. 'Gala' ingebracht door gebruik te maken van het merker-vrije systeem pMF1. Verschillende appel transformanten werden geselecteerd voor verdere karakterisering.

Micro-enten (micrografting) werd uitgevoerd om de 'in vitro' transformanten naar de kas over te brengen. Deze methode bleek de groei beter te bevorderen dan het gebruik van gewortelde 'in vitro' transformanten. Getransformeerde 'in vitro' appelscheuten werden geënt op een appelzaailing als onderstam. De micro-enten waren 4 tot 5 weken na het enten klaar voor vervolgonderzoek. In dit stadium werd jong blad verzameld voor isolatie van DNA en RNA. Southern hybridisatie werd uitgevoerd om het aantal kopieën van T-DNA insertie te bepalen. Hiervoor werd de selectiemerker *npt*II gebruikt als probe. De meeste transformanten (17) bleken een enkele T-DNA insertie te bevatten en zeven transformanten hadden twee T-DNA inserties. Vervolgens is de *HcrVf* gen-expressie in de getransformeerde lijnen bestudeerd met kwantitatieve RT-PCR (qRT-PCR) en gerelateerd aan de natuurlijke *HcrVf* expressie in het resistente ras 'Santana'. In het geval van de *HcrVf1* transformanten was de expressie verkregen door LP significant hoger dan door SP, terwijl voor de *HcrVf2*

transformanten geen significant verschil tussen SP en LP kon worden aangetoond. Beide *HcrVf1* en *HcrVf2* genen lieten de hoogste expressie zien wanneer ze gereguleerd werden door de appel rubisco promotor en terminator. Twee *HcrVf2* transformanten, LP*HcrVf2*-4 en P_{MdRbc}*HcrVf2*-12 lieten de hoogste expressie zien voor respectievelijk de cisgene en de intragene situatie. Bij de *HcrVf* transformanten werd geen significante correlatie waargenomen tussen het aantal genkopieën dat was geïnserteerd en het niveau van genexpressie (Chapter3).

Micro-geënte cv. 'Santana' (resistente controle met *HcrVf* verkregen door klassieke veredeling) en cv. 'Gala' (vatbare controle) scheutjes en verschillende micro-geënte appel transformanten werden getest voor schurftresistentie tegen *V. inaequalis* isolaat EU-B05. De vier topbladeren werden gebruikt voor inoculatie met *V. inaequalis*. Zeventien dagen na inoculatie werden de planten gescoord voor sporulatie, gebruik makend van een kwantitatieve schaal. Alle *HcrVf1* transformanten lieten volop sporulatie, gelijk aan het niveau van cv. 'Gala' zien, wat aangeeft dat *HcrVf1* geen resistentie geeft. Aan de andere kant lieten 10 van de 13 *HcrVf2* transformanten resistentie zien op een niveau dat statistisch niet verschilt van dat van cv. 'Santana'. Twee *HcrVf2* transformanten, LP*HcrVf2*-4 en P_{MdRbc}*HcrVf2*-12, lieten de beste resistentie zien. Er werd een negatieve correlatie gevonden tussen *HcrVf2* gen expressie en de mate van sporulatie, wat betekent dat wanneer de genexpressie toeneemt, de schimmelsporulatie afneemt (Chapter 4).

De verkregen resultaten in de schurfttoets werden gebruikt om *HcrVf1* en *HcrVf2* transformanten te selecteren en om het resistentiespectrum tegen verschillende isolaten van *V. inaequalis* vast te stellen. De planten werden geïnoculeerd met vier avirulente isolaten van het pathogeen en twee isolaten die virulent zijn voor het resistente ras 'Santana'. Twee topbladeren werden geïnoculeerd met sporen van de schimmel en de geïnoculeerde planten werden 21 dagen na inoculatie gescoord voor sporulatie. Alle *HcrVf1* transformanten lieten een sterke sporulatie zien bij alle gebruikte isolaten en ze gedroegen zich als ongetransformeerde cv. 'Gala'. De *HcrVf2* transformanten gedroegen zich als cv. 'Santana' wat aangeeft dat de resistentie van het *HcrVf* gen cluster alleen afkomstig is van *HcrVf2* (Chapter 5).

Om de duurzaamheid van schurftresistentie te bevorderen is het stapelen van verschillende resistentiegenen in appelrassen door klassieke veredeling of door genetische modificatie gewenst. Voor een cisgene of intragene benadering moeten nieuwe schurftresistentiegenen worden geïdentificeerd en gekloneerd. In 'Chapter 6' wordt beschreven hoe een nieuw schurftresistentiegen, *Vd3*, is geïdentificeerd en genetisch in kaart is gebracht in de resistente selectie 1980-015-025. Voor dit onderzoek werden de F1-nakomelingen van populatie 2000-012, die afkomstig zijn van een kruising tussen de

resistente en een vatbare selectie, gebruikt. Voor het genetisch in kaart brengen zijn voornamelijk DArT merkers toegepast. Andere bekende moleculaire merkers, zoals SSRs, P-136 (RAPD merker) en *Vf2ARD* (RGA merker) werden geselecteerd om de koppelingsgroepen te duiden. Het *Vd3* gen bevindt op koppelingsgroep 1, 1 cM ten zuiden van het *Vf* gen cluster, in afstotingsfase ten opzichte van *Vf*. Afstammingsgegevens geven aan dat selectie 1980-015-025 het *Vd3* gen geërfd heeft van de 'founder' accessie D3. Dit gen verschaft resistentie tegen het *Vf* virulente isolaat EU-NL24, dat de resistentie van *Vf* en *Vg* heeft doorbroken. *Vd3* geeft echter geen resistentie tegen andere getoetste isolaten (Chapter 6).

De in dit proefschrift beschreven resultaten zijn van praktisch belang. Cisgenese of intragenese kan worden toegepast om polygene resistentie tegen schurft in appel te brengen, zonder de 'linkage drag' problemen welke optreden in klassieke veredeling. De eerste potentiële cisgene schurftresistente planten van cv. 'Gala' met het *HcrVf2* gen die we ontwikkeld hebben kunnen geteeld worden in gebieden die vrij zijn van virulente isolaten. De cisgenese benadering is essentieel voor het versneld verbeteren van een gewas zoals appel waarvoor in de conventionele veredeling meerdere decennia nodig zijn.

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SAMEER GURURAJ JOSHI

Curriculum Vitae

Sameer Gururaj Joshi was born on 23rd September 1978 in Jamkhandi, Karnataka, India. He received his Bachelor degree (B.Sc.) in Horticulture from University of Agricultural Sciences, Bangalore, India in 2000. He obtained his Master degree (M.Sc.) in Horticulture with specialization in Pomology from University of Agricultural Sciences, Bangalore, India in 2003. He worked as Research Associate in the Division of Vegetable Crops at Indian Institute of Horticulture Research, Bangalore, India from 2004-2006. In 2006 he started his Ph.D. research at Plant Research International (PRI), Wageningen University and Research Centre under the supervision of Prof. Dr. Ir. Evert Jacobsen, Dr. Frans A. Krens and Dr. Henk J. Schouten. In his Ph.D. he worked on “Towards durable resistance to apple scab using cisgenes” which resulted in the outcome of this thesis.

List of Publications

Sameer Joshi, José Miguel Soriano, Jan Schaart, Giovanni Antonio Lodovico Broggini, Iris Szankowski, Evert Jacobsen, Frans Krens, Henk J. Schouten (2009) Approaches for Development of Cisgenic Apples. *Transgenic Plant Journal* 3 (Special Issue 1), 40-46

J.M. Soriano*, **S.G. Joshi***, M. van Kaauwen, Y. Noordijk, R. Groenwold, B. Henken, W.E. van de Weg and H.J. Schouten (2009) Identification and mapping of the novel apple scab resistance gene *Vd3*. *Tree Genetics and Genomes* 5, 475-482

* JMS and SGJ contributed equally to this work

S.G. Joshi, J.G. Schaart, R. Groenwold, E. Jacobsen, H.J. Schouten, F.A. Krens. Functional analysis and expression profiling of *HcrVf1* and *HcrVf2* for development of scab resistant cisgenic and intragenic apples. *Manuscript in preparation*

S.G. Joshi, J.G. Schaart, R. Groenwold, E. Jacobsen, F.A. Krens, H.J. Schouten. Induction and expression studies of *HcrVf* genes in apple. *Manuscript in preparation*

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: **Sameer Gururaj Joshi**
Date: **27 May 2010**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Stacking functional expressed apple genes for durable resistance to apple scab.	Jun 04, 2007
► **Writing or rewriting a project proposal**	
Stacking functional expressed apple genes for durable resistance to apple scab.	Nov 06, 2007
► **Writing a review or book chapter**	
Invited mini review submitted to Global Science Book:Approaches for development of cisgenic apples	Nov 23, 2009
► **MSc courses**	
Plant Biotechnology	Feb 02, 2007
Genetic Analysis Tools and Concepts (GATC)	May 31, 2007
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*19.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS Ph.D. student day 2006, Wageningen University	Sep 19, 2006
EPS Ph.D. student day 2007, Wageningen University	Sep 13, 2007
EPS Ph.D. student day 2009, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS theme symposium Theme 4, Nijmegen	Dec 11, 2009
EPS theme symposium Theme 1, Wageningen	Jan 28, 2010
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren 2008	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren 2009	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren 2010	Apr 19-20, 2010
► **Seminars (series), workshops and symposia**	
EPS flying seminar by Prof Jim Carrington	Mar 26, 2007
EPS flying seminar by Prof Scott Poethig	Sep 24, 2007
EPS seminar by Prof. Jian-Kang Zhu	Nov 03, 2008
EPS seminar by Prof. Maria L. Badenes	Dec 18, 2008
► **Seminar plus**	
► **International symposia and congresses**	
Biotechfruit2008, Dresden, Germany	Sep 01-05, 2008
Cost Action 864 meeting WG 4 on pome fruits September 2007	Sep 05-06, 2007
The XIV Congress on Molecular plant microbe interaction, Quebec, Canada	Jul 19-23, 2009
XVIII Plant and Animal Genome Conference, San Diego, CA, USA	Jan 09-13, 2010
Cost Action 864 meeting WG 4 on pome fruits February 2010	Feb 11-12, 2010
► **Presentations**	
Biotechfruit2008, Dresden- Poster presentation	Sep 01-05, 2008
The XIV Congress on Molecular plant microbe interaction, Quebec, Canada- Poster presentation	Jul 19-23, 2009
XVIII Plant and Animal Genome Conference, San Diego, CA, USA- Poster presentation	Jan 09-13, 2010
► **IAB interview**	Dec 05, 2008
► **Excursions**	
Subtotal Scientific Exposure	*12.2 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Summer school Ön the evolution of plant pathogen interaction from principles to practice	Jun 18-20, 2008
Gateway to Gateway technology	Nov 17-21, 2008
Course:Quantitave PCR techniques, Breda, NL	Jul 08-10, 2009
Bioinformatics-A user's approach	Mar 15-19, 2010
► **Journal club**	
Literature discussions and work discussions at plant breeding	2006-2010
► **Individual research training**	
Subtotal In-Depth Studies	*6.4 credits**

4) Personal development	*date*
► **Skill training courses**	
Course:Techniques for writing and presenting scientific papers	Dec 15-18, 2009
► **Organisation of PhD students day, course or conference**	
Organisation of Cost meeting WG 4 on pome fruits	Feb 11-12, 2010
► **Membership of Board, Committee or PhD council**	
EPS Ph.D. council member	Jan-Jun 2010
Subtotal Personal Development	*3.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**41.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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